

**CARDIOVASCULAR AND RESPIRATORY EFFECTS OF EXPOSURE
TO EXPERIMENTAL MODELS OF OBSTRUCTIVE SLEEP
APNOEA-RELATED INTERMITTENT HYPOXIA**

By

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ABSTRACT

Obstructive sleep apnoea (OSA) is a common chronic condition characterised by repetitive nocturnal upper airway collapse that evokes intermittent hypoxia (IH). Although animal research has demonstrated a causal relationship between IH and cardiovascular disease there is a relative paucity of human research. Using a variety of different models of OSA we investigated the effects of IH on respiratory control and oxidative stress. In addition, we attempted to use Doppler ultrasound to investigate the effects of hypoxic airway occlusions on the pulmonary circulation but concluded that it is not a feasible alternative to invasive catheterisation. In a number of studies we showed that the expression of respiratory plasticity following IH is only evident when arterial levels of CO₂ are raised above normal levels. Furthermore, in stark contrast to previous findings in animals, exposure to acute continuous hypoxia also evokes respiratory plasticity in humans. In addition, we showed that combined postprandial hyperglycaemia and hyperlipidaemia augments the degree of oxidative stress during IH. Finally, we demonstrated that IH accentuates the magnitude of postprandial hyperglycaemia. These studies demonstrate the complexity of respiratory control in humans and they highlight significant species differences. Furthermore, they highlight a fascinating synergy between IH and the postprandial state.

THESIS OVERVIEW

The work presented in this thesis investigates the effects of acute exposure to intermittent hypoxia (IH) in relation to its common occurrence in the chronic disease, obstructive sleep apnoea. IH appears to be a stimulus that is capable of eliciting cardiovascular disease through mechanisms that are currently not fully understood. However, in contrast with its potential to mediate disease, acute exposure to IH is capable of inducing respiratory plasticity that could potentially be harnessed to treat diseases including obstructive sleep apnoea.

This thesis has investigated the possible detrimental health effects of IH and its ability to elicit respiratory plasticity. For simplicity, in both the general introduction (chapter 1) and general discussion (chapter 8) respiratory plasticity will be discussed separately from the detrimental health effects of IH.

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TABLE OF CONTENTS

1	CHAPTER 1 – GENERAL INTRODUCTION	1
1.1	Obstructive sleep apnoea.....	1
1.1.1	General	1
1.1.2	Pathophysiology of OSA	2
1.1.3	Epidemiology	3
1.1.4	Obstructive sleep apnoea and cardiovascular disease.....	4
1.2	Intermittent hypoxia	9
1.2.1	General	9
1.2.2	IH-mediated cardiovascular disease	10
1.3	Oxidative stress.....	14
1.3.1	General	14
1.3.2	IH-induced oxidative stress	16
1.3.3	Mechanisms of IH-induced oxidative stress	18
1.4	IH-induced oxidative stress during the postprandial state	23
1.4.1	Simultaneous exposure to IH and postprandial hyperglycaemia	24
1.4.2	Simultaneous exposure to IH and postprandial hyperlipidaemia	27
1.5	IH-mediated respiratory plasticity	29
1.5.1	General	29
1.5.2	Why investigate IH-mediated respiratory plasticity?	33
1.5.3	Respiratory LTF in animals	38

1.5.4	Respiratory LTF in humans.....	43
1.5.5	Progressive augmentation of the HVR.....	48
1.5.6	Summary of respiratory plasticity	53
1.6	Aims of this thesis and proposed studies	54
1.6.1	Primary questions investigated in this thesis	55
2	CHAPTER 2 – GENERAL METHODS.....	57
2.1	Gas control.....	57
2.1.1	Dynamic end-tidal forcing.....	58
2.2	Development of a novel airway occlusion model of OSA	60
2.2.1	General	60
2.2.2	Existing models of OSA and identification of potential improvements	61
2.2.3	Model design	69
2.2.4	Model evaluation and implications for future research.....	79
2.3	Echocardiography	82
2.3.1	Two-dimensional cardiac imaging.....	83
2.3.2	Doppler ultrasound.....	85
2.3.3	Doppler modes	87
2.3.4	Cardiac views	90
2.3.5	Estimation of SPAP.....	92
2.3.6	Cardiac output	93
3	CHAPTER 3 - ASSESSING PULMONARY ARTERIAL PRESSURE DURING AIRWAY OCCLUSIONS USING DOPPLER ULTRASOUND	96
3.1	Introduction	96

3.2	Methods	105
3.3	Results	111
3.4	Discussion	116
4	CHAPTER 4 - MANIFESTATION OF RESPIRATORY PLASTICITY	
	FOLLOWING HYPOXIC AIRWAY OCCLUSIONS IN AWAKE HUMANS	120
4.1	Introduction	120
4.2	Methods	123
4.3	Results	128
4.4	Discussion	138
5	CHAPTER 5 - LONG-TERM FACILITATION OF VENTILATION	
	FOLLOWING ACUTE CONTINUOUS HYPOXIA IN AWAKE HUMANS DURING	
	SUSTAINED HYPERCAPNIA.....	145
5.1	Introduction	145
5.2	Methods	148
5.3	Results	155
5.4	Discussion	165
6	CHAPTER 6 - USE OF AN ANIMAL AIRWAY OCCLUSION MODEL OF OSA	
	TO INVESTIGATE WHETHER SIMULTANEOUS HYPERGLYCAEMIA	
	AUGMENTS IH-INDUCED OXIDATIVE STRESS	179
6.1	Introduction	179
6.2	Methods	183
6.3	Results	192
6.4	Discussion	199

7	CHAPTER 7 - SIMULTANEOUS EXPOSURE TO ACUTE INTERMITTENT HYPOXIA AND COMBINED POSTPRANDIAL HYPERGLYCAEMIA AND HYPERLIPIDAEMIA IN HEALTHY HUMANS	207
7.1	Introduction	207
7.2	Methods	211
7.3	Results	220
7.4	Discussion.....	231
8	CHAPTER 8 - GENERAL DISCUSSION	244
8.1	Development of an airway occlusion model of OSA.....	244
8.2	Investigating the effects of acute IH as it occurs in OSA on pulmonary arterial pressure.....	245
8.3	IH and oxidative stress	247
8.4	Respiratory plasticity	250
8.4.1	Respiratory plasticity in OSA and the importance of CO ₂	250
8.4.2	Separating respiratory LTF from progressive augmentation of the HVR	252
8.4.3	Future use of pharmaceutical aids to mimic IH-induced respiratory LTF	253
8.4.4	Hypoxic induced ventilatory LTF is neither pattern sensitive nor maintained by the CB in awake humans.....	255
8.5	Future research.....	256
8.5.1	Future research into respiratory plasticity.....	256

8.5.2	Future research investigating the effects of IH on oxidative stress during hyperglycaemia.....	257
9	REFERENCES.....	259

LIST OF FIGURES

Figure 1.1: Raw data from a polysomnography showing various physiological events during an apnoeic cycle.....	8
Figure 1.2: A schematic showing the responsible mechanisms for increased ROS generation during ischaemia-reperfusion injury.	20
Figure 1.3: Reactive oxygen species production ($\Delta I(nA)$) during cerebral ischaemia-reperfusion injury..	27
Figure 1.4: Schematic illustrating IH-induced respiratory modulation and respiratory plasticity.	32
Figure 1.5: Schematic demonstrating the proposed responsible mechanistic pathway for phrenic LTF.	43
Figure 2.1: Original data showing S_aO_2 , PET_{O_2} and tidal volume during exposures to 100% inspired N_2 in two participants.....	66
Figure 2.2: A photograph of the experimental setup used to induce airway occlusions.	76
Figure 2.3: Original record for one participant showing air flow and intrathoracic pressure during successive airway occlusions.	77
Figure 2.4: Original recordings from one participant showing breath-by-breath PET_{O_2} , PET_{CO_2} , TV, S_aO_2 and systemic BP during a sequence of airway occlusions with and without hypoxia.	81
Figure 2.5: Illustration of the scan lines from an ultrasonography transducer.	85

Figure 2.6: A spectral waveform of tricuspid regurgitation measured in continuous wave Doppler mode.	89
Figure 2.7: Apical four-chamber, five-chamber and parasternal long-axis views of the heart with ultrasound.....	91
Figure 2.8: Apical five-chamber view illustrating the tricuspid regurgitation jet (1) as visualised in colour Doppler mode.	92
Figure 2.9: Ejection of blood through the aortic valve as visualised in colour Doppler mode using a five-chamber view.	94
Figure 2.10: A PWD spectral trace of flow through the aortic valve.....	95
Figure 3.1: Average transmural PAP (tPAP) over a range of intrathoracic pressures in four patients with OSA..	98
Figure 3.2: Systolic pulmonary arterial pressure at baseline and in recovery from airway occlusions.....	114
Figure 3.3: Cardiac output at baseline and in recovery from airway occlusions. .	114
Figure 3.4: Heart rate at baseline and in recovery from airway occlusions.	115
Figure 3.5: Stroke volume at baseline and in recovery from airway occlusions...	115
Figure 4.1: Minute ventilation during room air baseline, and room air recovery, following one hour hypoxic airway occlusions or unrestricted breathing.....	132
Figure 4.2: PET_{CO_2} and PET_{O_2} averaged for every minute during the AHVR test.	133
Figure 4.3: Averaged minute ventilation for isocapnic euoxic baseline, hypoxia and euoxic recovery of the AHVR test.	134

Figure 4.4: Minute ventilation during room air baseline and isocapnic euoxic baseline of the AHVR test.....	135
Figure 4.5: Systolic pulmonary arterial pressure during the AHVR test.....	136
Figure 4.6: Averaged cardiac output, heart rate and stroke volume during the AHVR test.	137
Figure 5.1: Schematic demonstrating performed protocols.	154
Figure 5.2: Minute averages for PET_{O_2} and PET_{CO_2} for each trial.....	158
Figure 5.3: Minute ventilation throughout Trial 1.	161
Figure 5.4: Minute ventilation throughout Trial 2.	162
Figure 5.5: Minute ventilation throughout Trial 3.	163
Figure 5.6: Change in minute ventilation from hypercapnic baseline to the final quarter of hypercapnic recovery.	164
Figure 5.7: Hyperoxia-induced reduction in minute ventilation.	164
Figure 6.1: Glucose infusion rates and blood glucose levels.....	195
Figure 6.2: Averaged plasma levels of malondialdehyde-protein adducts.....	196
Figure 6.3: Averaged plasma levels of nitrotyrosine.....	196
Figure 6.4: Averaged antioxidant capacity of plasma.	197
Figure 6.5: Averaged minute ventilation during baseline and recovery room air.	197
Figure 6.6: Averaged minute ventilation during hypoxia at baseline and recovery	198
Figure 6.7: Averaged mean arterial pressure during baseline and recovery room air.....	198

Figure 7.1: Schematic illustrating the protocol used for experimental trials.....	219
Figure 7.2: Levels of plasma lipid peroxidation at baseline and recovery.....	223
Figure 7.3: Levels of plasma protein carbonylation at baseline and recovery.	223
Figure 7.4: Plasma antioxidant capacity at baseline and recovery.	224
Figure 7.5: Correlation between the change in plasma lipid peroxidation and antioxidant capacity.	224
Figure 7.6: Plasma glucose levels over three hours.....	229
Figure 7.7: Plasma glucose three-hour incremental area under the curve.	229
Figure 7.8: Plasma triglyceride levels over three hours.....	230
Figure 7.9: Plasma triglyceride three-hour incremental area under the curve.	230
Figure 7.10: Schematic demonstrating how hyperglycaemia may increase xanthine oxidase generated ROS during ischaemia-reperfusion injury or IH.	236

LIST OF TABLES

Table 2.1: Timings of when occluded inspiratory efforts were to be performed during each airway occlusions and the desired negative intrathoracic pressures.	75
Table 4.1: PET_{CO_2} , PET_{O_2} and S_aO_2 values during room air baseline and room air recovery following hypoxic airway occlusions or unrestricted breathing.	132
Table 4.2: Averaged PET_{CO_2} , PET_{O_2} and S_aO_2 values for isocapnic euoxic baseline, hypoxia and euoxic recovery of the AHVR test.	134
Table 5.1: Averaged PET_{O_2} , PET_{CO_2} and S_aO_2 values for eucapnic baseline and recovery.	159
Table 5.2: Averaged PET_{O_2} , PET_{CO_2} and S_aO_2 values for hyperoxic exposures during eucapnic baseline and recovery.	159
Table 5.3: Averaged PET_{O_2} , PET_{CO_2} and S_aO_2 values for hypercapnic baseline and recovery.	160
Table 5.4: Averaged PET_{O_2} , PET_{CO_2} and S_aO_2 values for hyperoxic exposures during hypercapnic baseline and recovery.	160
Table 7.1: Averaged ventilatory measurements during baseline and recovery. ..	228
Table 7.2: Averaged ventilatory measurements during early and late hypoxic episodes.	228

ABBREVIATIONS

AHI	apnoea hypopnoea index
AHVR	acute hypoxic ventilatory response
ANOVA	analysis of variance
BCA	bicinchoninic acid method
BDNF	brain-derived neurotrophic factor
BF	breathing frequency
BMI	body mass index
CB	carotid body
CH	continuous hypoxia
CNS	central nervous system
COPD	chronic obstructive pulmonary disease
CPAP	continuous positive airway pressure
CSA	central sleep apnoea
CWD	continuous wave Doppler
DEF	dynamic end-tidal forcing
ECG	electrocardiography
ELISA	enzyme-linked immunosorbent assay
EDTA	ethylenediaminetetraacetic acid
FRAP	ferric reducing ability of plasma
HIF	hypoxia-inducible factor
HR	heart rate

HVR	hypoxic ventilatory response
iAUC	incremental AUC
IH	intermittent hypoxia
IRI	ischemia-reperfusion injury
LPO	lipid peroxidation
LTF	long-term facilitation
LSD	Fisher's least significant difference
MAP	mean arterial pressure
MDA	malondialdehyde-protein adducts
NADPH oxidase	nicotinamide adenine dinucleotide phosphate-oxidase
OSA	obstructive sleep apnoea
PK-C	protein kinase C
PUFA	polyunsaturated fatty acids
P _A	alveolar partial pressure
P _a	arterial partial pressure
PAP	pulmonary arterial pressure
P _{ET}	end-tidal partial pressure
P _i	inspired partial pressure
PWD	pulsed wave Doppler
Q	cardiac output
RA	room air
RAP	right atrium pressure

ROS	reactive oxygen species
RNS	reactive nitrogen species
S _a O ₂	arterial oxygen saturation
SOD	superoxide dismutase
SPAP	systolic pulmonary arterial pressure
SV	stroke volume
TAG	triglyceride
tPAP	transmural pulmonary arterial pressure
T2DM	type two diabetes mellitus
TV	tidal volume
UANP	upper airway negative pressure
\dot{V}_E	minute ventilation
VTI	velocity time integral
5-HT	5-hydroxytryptamine or serotonin

1 CHAPTER 1 – GENERAL INTRODUCTION

1.1 Obstructive sleep apnoea

1.1.1 General

Sleep apnoea is a common chronic disorder in which individuals experience frequent and abnormally long restrictions in breathing during sleep. There are two distinct forms of sleep apnoea: obstructive sleep apnoea (OSA) and central sleep apnoea. OSA is characterised by repetitive complete or partial obstruction of the upper airway during sleep that prevents (apnoea) or reduces (hypopnoea) airflow despite continued inspiratory effort. This is followed by transient incomplete arousal that allows restoration of upper airway patency. Central sleep apnoea like OSA involves both apnoeas and hypopnoeas but unlike OSA where inspiratory efforts continue against an occluded airway, respiratory effort is absent (apnoea) or diminished (hypopnoeas) in central sleep apnoea. Although sleep apnoea patients suffer from predominantly one of the two forms of sleep apnoea it is not uncommon for patients to experience a combination of obstructive and central apnoeas/hypopnoeas termed “complicated sleep apnoea”. This thesis will focus

predominantly on OSA but due to the common overlap between the two forms and because both elicit IH, central sleep apnoea will be discussed where appropriate.

1.1.2 Pathophysiology of OSA

During normal breathing air is drawn into the alveoli down a pressure gradient generated by contraction of respiratory muscles (diaphragm and intercostals) that expand the thorax and lower alveolar pressure below atmospheric pressure. Because of this pressure gradient between the alveoli and the external environment the pressure inside the upper airway (e.g. mouth and throat) is transiently negative during inspiration. The surrounding tissue of the upper airway is not a rigid structure and thus, it is vulnerable to collapse during these periods of negative pressure. To prevent this from occurring muscles surrounding the soft tissue of the upper airway contract during inspiration to maintain its patency. These muscles receive neural activation in sync with central respiratory pattern generation and from mechanoreceptor feedback within the airway that responds to negative pressures (Edwards and White, 2011). OSA patients commonly have an anatomically narrow upper airway that increases resistance to airflow and subsequently augments the negative pressure in the upper airway during inspiration making it more vulnerable to collapse (Schwab et al., 2003). However, it has been shown that during wakefulness OSA patients demonstrate greater basal activity of the genioglossus muscle compared to controls. The genioglossus is the primary muscle of the upper airway dilator group and thus during wakefulness in these patients the upper airway does not collapse (Mezzanotte et al., 1992).

Although the mechanisms are not fully understood, during sleep the activity of the upper airway dilator muscles is reduced and thus the neuromuscular compensation present during wakefulness in OSA patients is lost and the upper airway is prone to collapse (Edwards and White, 2011). Upper airway patency is only restored following an apnoea or hypopnoea by partial arousal from sleep that increases the tone of these upper airway dilator muscles. It is not fully known what triggers arousal from sleep but it is likely to be in part a response to an increase in chemoreceptor drive due to increased levels of arterial partial pressure of CO₂ (PaCO₂) and decreased levels of arterial partial pressure of O₂ (PaO₂) (Sforza et al., 1998). Furthermore, it may also be in response to the progressive increase in respiratory muscle activity during the increasing frequency and magnitude of occluded inspiratory efforts (Sforza et al., 1998).

1.1.3 Epidemiology

In order to diagnose OSA a sleep study (polysomnography) is performed where numerous measurements such as airflow and arterial O₂ saturation (S_aO₂) are made continuously. A standardised criterion has been developed to determine the number of apnoeas and hypopnoeas experienced by the patients by assessing the recorded measurements. Firstly, an apnoea is defined as the complete absence of airflow for over 10 seconds. Secondly, a hypopnoea is defined as a 50% or more reduction in airflow for over 10 seconds or a noticeable reduction in airflow with a reduction in S_aO₂ of more than 4%. The severity of OSA is then determined by the

number of apnoeas and hypopnoeas per hour (i.e. apnoea/hypopnoea index: AHI). An AHI of 5-15 is classed as mild OSA, an AHI of 15-30 as moderate and an AHI of over 30 as severe (American Academy of Sleep Medicine).

OSA is now a highly prevalent condition in the developed world. A relatively conservative figure compared to numerous other reports is that 4% of middle-aged men and 2% of middle-aged women suffer from some degree of OSA (Young et al., 1993). However, the actual prevalence of OSA may be much higher as a study estimated that only 18% of men with moderate to severe OSA were clinically diagnosed (Young et al., 1997). The greatest risk factor for OSA is an anatomically narrow upper airway. This increases resistance to airflow and subsequently augments the negative pressure during inspiration promoting upper airway collapse (Schwab et al., 2003). A number of physiological characteristics such as, tonsillar hypertrophy, tongue shape, and a short neck can cause a narrowing of the upper airway and thus increase the risk of OSA (Azagra-Calero, 2012). However, by far the greatest risk factor for OSA is obesity. Obese individuals are prone to having increased adipose tissue and soft tissue edema in the neck region which causes a narrowing of the upper airway and a subsequent increase in upper airway resistance.

1.1.4 Obstructive sleep apnoea and cardiovascular disease

There is unison between large-scale epidemiology studies showing a greater prevalence of cardiovascular disease in OSA patients than the general population

(Somers et al., 2008). However, demonstrating a causal link between OSA and cardiovascular disease is challenging due to the high prevalence of comorbidities such as, obesity and diabetes. However, studies reporting reduced rates of mortality in OSA patients who received treatment (tracheotomy or continuous positive airway pressure - CPAP) vs. those that didn't strongly suggests a causative link (He et al., 1988, Partinen et al., 1988).

Each apnoeic event involves a complex set of acute physiological stressors and cardiovascular responses, all of which may chronically be capable of facilitating the development and progression of cardiovascular disease in OSA patients (Golbin et al., 2008). Figure 1.1 is an original polysomnogram from a patient with OSA and it illustrates a number of these different physiological events that occur during an apnoeic cycle. It shows that whilst the upper airway is collapsed inspiratory efforts continue and the development of a substantial negative intrathoracic pressure is associated with each such effort. The magnitude of the negative intrathoracic pressure that is generated with each effort gradually increases throughout the apnoea. The physiological effects of these negative intrathoracic pressures are discussed in detail in chapter 2 but in brief, they transiently increase venous return (Moreno et al., 1967) but simultaneously decrease stroke volume (Tolle et al., 1983, Summer et al., 1979, Orban et al., 2008). This leads to a congestion of blood in the pulmonary circulation that transiently elevates pulmonary arterial pressure (PAP) (Marrone et al., 1994). Figure 1.1 also shows systemic BP steadily increases during each apnoea that peaks upon arousal. It has been shown in

humans that inhibiting sympathetic activity with ganglionic blockade abolishes the pressor response to Mueller manoeuvres and breath-holds suggesting that the elevation in systemic blood pressure during an apnoea is mediated completely by increased sympathetic activity (Katragadda et al., 1997). This increase in sympathetic activity is primarily, but not solely, driven by chemoreflex stimulation and thus may also depend on a loss of mechanoreceptor activity during occluded apnoeas (e.g. lung reflexes) (Chen et al., 1998). Each apnoea or hypopnoea is terminated by a sudden arousal from sleep that causes a further elevation in sympathetic activity (Odonnell et al., 1996). Immediately post apnoea there is a brief period of tachycardia as shown by the ECG trace in the in figure 1.1 (Stoohs and Guilleminault, 1992, Zwillich et al., 1982). This is likely to be in response to increased activation of lung reflexes as it occurs parallel to a period of hyperventilation and/or due to elevated sympathetic activity that peaks post apnoea. In addition to the aforementioned physiological events, apnoeas evoke a significant increase in PaCO_2 that rapidly returns to or below eucapnic levels following the post apnoeic hyperventilation (Lanphier and Rahn, 1963). Although chronic exposure to all of the aforementioned physiological stressors and cardiovascular responses could theoretically in part mediate the development of cardiovascular disease the most likely stimulus is thought to be IH (Levy et al., 2008, Foster et al., 2007b). During an apnoea the cessation in alveolar ventilation causes a gradual decline in S_aO_2 thus exposing patients to whole body hypoxia which can be of a significant severity and occurs simultaneous to all the other

physiological events shown in figure 1.1. The partial pressure of arterial blood (PaO_2) is rapidly restored during the resumption of breathing following each apnoea which generates the intermittent pattern of hypoxia that is unique to sleep apnoea.

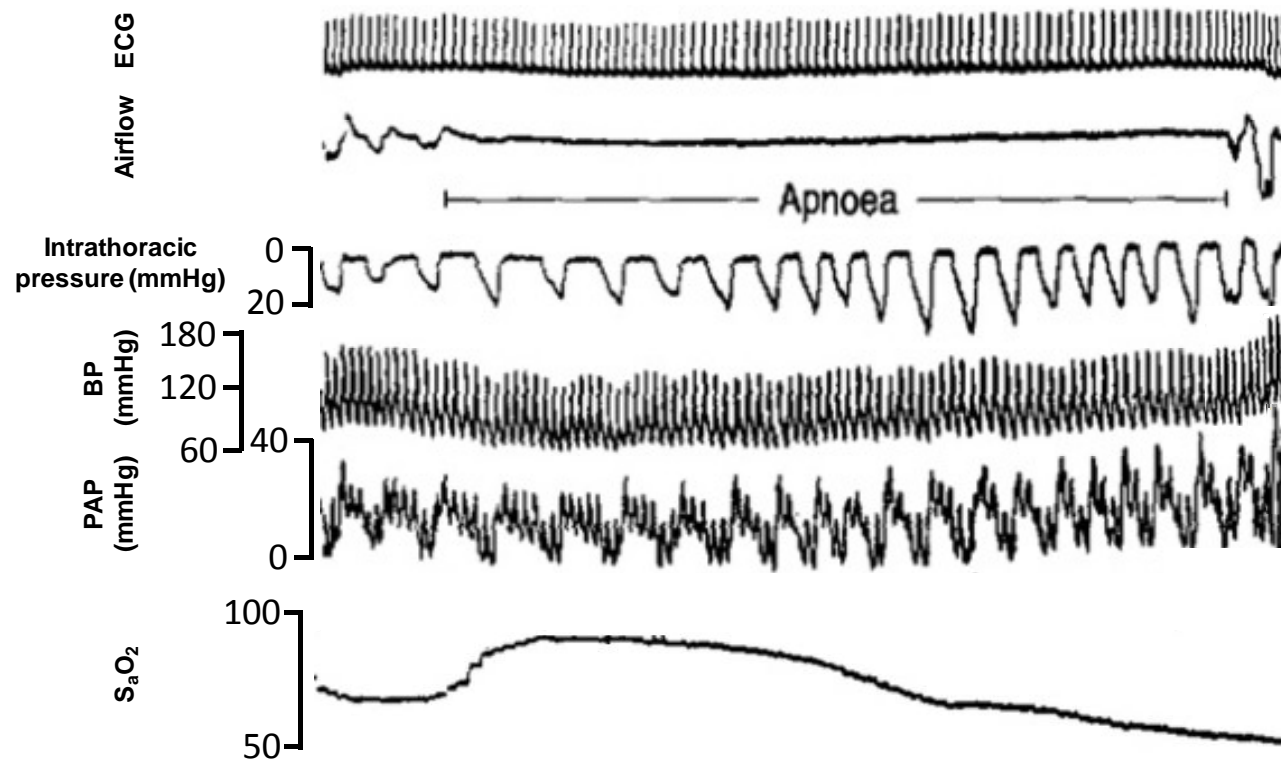


Figure 1.1: Raw data from a polysomnography showing various physiological events during an apnoeic cycle: Channel 1, (ECG) showing significant tachycardia immediately post apnoea; Channel 2, (Airflow) showing complete absence of airflow during airway occlusion; Channel 3, (Intrathoracic pressure) showing negative intrathoracic pressure during continued inspiratory efforts; Channel 4, (BP – systemic blood pressure) showing a gradual elevation in systemic BP during the apnoea that peaks during resumption of breathing; Channel 5, (PAP - pulmonary arterial pressure) showing transient swings in PAP during each occluded inspiratory effort and a gradual rise throughout the apnoea, and Channel 6, (S_aO_2 - arterial O_2 saturation) showing a gradual development of hypoxia. Adapted from Sajkov and McEvoy (2009).

1.2 Intermittent hypoxia

1.2.1 General

Although all mammalian cells have the capability of transiently generating cellular energy without O_2 (anaerobic respiration), survival of almost all mammalian cells is reliant on a continuous supply of O_2 for aerobic respiration. Therefore, the human body has evolved to develop intricate structures and processes to transport O_2 from the atmosphere to all cells (e.g. breathing, circulation etc). Following upper airway collapse this highly evolved system fails at the first hurdle as O_2 cannot be drawn into the alveoli. Therefore, the partial pressure of O_2 in the alveoli (PA_{O_2}) gradually falls during an apnoea, resulting in a subsequent drop in Pa_{O_2} . Due to the sigmoidal shape of the oxygen-haemoglobin dissociation curve this initially has little effect on S_aO_2 and thus tissue O_2 delivery is little affected. Although, there is substantial variation in apnoea duration within and between individuals it is extremely common for apnoeas to exceed 20 seconds. During apnoeas of this duration Pa_{O_2} is often reported to fall as low as 40-50 mmHg equating to S_aO_2 of 75-85% with nadirs in S_aO_2 falling into the low 50s in patients with severe OSA (Sforza et al., 1998). Therefore, OSA patients are frequently exposed to whole body hypoxia as would be experienced at a considerable altitude (i.e. ~5000 meters). However, unlike continuous hypoxia (CH) experienced at altitude and in chronic conditions such as chronic obstructive pulmonary disease (COPD) the S_aO_2 is rapidly restored following each apnoea and thus OSA patients experience

IH. Evidence has been emerging in the last couple decades demonstrating how exposure to IH may evoke significant pathological effects.

1.2.2 IH-mediated cardiovascular disease

The current treatment of choice for OSA is CPAP and studies using it have been able to demonstrate the reversal of many health complications of OSA such as, systemic hypertension (Somers et al., 2008), pulmonary hypertension (Sajkov and McEvoy, 2009), endothelial dysfunction (Foster et al., 2007a, El Solh et al., 2007, Ip et al., 2004) and elevated oxidative stress (Barcelo et al., 2000, Lavie et al., 2004, Mancuso et al., 2012). However, because CPAP inhibits upper airway collapse it prevents not only exposure to IH but all the other physiological events that occur during occluded apnoeas. Therefore, in these studies IH cannot be identified as the stimulus that is responsible for the development of cardiovascular disease in OSA patients. Thus, scientists have opted to utilise an alternative approach to investigate the possible pathological effects of IH. To this end, numerous studies have been undertaken where animals have been exposed to IH independently of any other OSA-related stimuli by intermittently reducing the partial pressure of inspired O₂ (P_IO₂) to evoke a similar pattern and magnitude of hypoxaemia that is experienced during frequent apnoeas in OSA. Daily exposure to IH for several months evokes systemic hypertension (Brooks et al., 1997, Fletcher et al., 1992) pulmonary hypertension (Fagan, 2001, McGuire and Bradford, 2001) elevated sympathetic activity (Sica et al., 2000), atherosclerosis

(Savransky et al., 2007) and myocardial dysfunction (Williams et al., 2010, Chen et al., 2005) in animals. These animal studies have certainly highlighted the likelihood that IH may mediate the increased risk of cardiovascular disease in OSA. However, although this scientific approach benefits from separating the hypoxic element from other stimuli that occur in OSA so that the effects of hypoxia *per se* can be isolated, it also carries a significant limitation. Studies of this nature make the assumption that the body responds to intermittent reductions in P_{IO_2} in the same way as it would when IH occurs during occluded apnoeas. The possible synergy of IH and other physiological stressors and cardiovascular responses that occur during an occluded apnoea are thus ignored. Although speculative it would seem highly likely that IH may have a more detrimental effect on health when exposure is simultaneous with these various physiological stressors and cardiovascular responses than it would have independently. For example, systemic blood pressure does not increase during hypoxia when it is evoked by a reduction in P_{IO_2} but during an occluded apnoea hypoxia is the predominant mechanism responsible for causing the significant elevation in systemic blood pressure (Schneider et al., 2000). It is plausible that hypoxia may be more detrimental to cells of the cardiovascular system (specifically myocardial and endothelial cells) whilst simultaneously being under stress from the elevated systemic blood pressure. Interestingly, IH has been shown to impair endothelial function in OSA patients through increases in oxidative stress (El Solh et al., 2006) and it is not too far reached to postulate that a damaged vascular endothelium will be less able to

cope with apnoea-induced blood pressure elevations. Furthermore, during the negative intrathoracic pressure generated during each occluded inspiratory effort left ventricular contractility increases in order to maintain left ventricular pressure and prevent a fall in stroke volume (SV). Therefore, myocardial O₂ consumption increases (Simpson et al., 2008) at a time when there is considerable arterial hypoxia. Indeed, in anaesthetised rats exposed to airway occlusions that evoked significant negative intrathoracic pressures and hypoxia, myocardial dysfunction developed after as little as three hours of exposure (Simpson et al., 2008). Furthermore, although restoration of upper airway patency removes the burden on the heart of having to increase left ventricular contractility, tachycardia induced by the post apnoea hyperventilation and/or increase in sympathetic activity (Stoohs and Guilleminault, 1992, Zwillich et al., 1982) occurs simultaneous to the nadir in S_aO₂. As a result myocardial O₂ consumption is again elevated during a period of severe hypoxia (Simpson et al., 2008). Finally, exposure to intermittent reductions in P_IO₂ is associated with enhanced ventilation and subsequent hypocapnia. In contrast, during an occluded apnoea the absence of alveolar ventilation elicits significant hypercapnia. This could have substantial implications on the effect that IH has on the pulmonary circulation as hypocapnia is known to cause vasodilatation (Balanos et al., 2003) and thus may offset the pathological effects of hypoxic pulmonary vasoconstriction (HPV).

Despite a large number of studies having been performed over the last two decades where animals were exposed to acute and chronic IH there is a relative paucity of research undertaken in humans. Although the findings from these animal studies goes some way to elucidating the possible detrimental health effects of OSA-related IH, there are well known cardiovascular, ventilatory and metabolic differences between species and thus there is a need for human studies to be performed. The methodological approach of replicating IH that occurs in OSA by intermittently reducing the $P_{I_{O_2}}$ can easily and safely be replicated in human research. However, as discussed previously, evoking IH in such a way may not truly replicate the full extent of the pathological effects of IH as experienced in OSA. This is less of a limitation in animal research where animals can be exposed to IH for several months, but due to the ethical restraints of chronic exposures in humans it is important that acute studies investigate the full magnitude of any pathological consequence of IH. Thus, an initial aim of this thesis was to design a model of OSA whereby awake humans could be exposed to IH simultaneous to as many other components of OSA. We hoped this model could then be used in studies throughout and beyond this thesis to better investigate the effects of IH on various physiological parameters such as, ventilation, pulmonary and systemic haemodynamics, oxidative stress etc. A detailed description of the design of this model follows in chapter 3.

1.3 Oxidative stress

1.3.1 General

Following the initial findings from animal studies that demonstrated the development of cardiovascular disease following chronic exposure to IH, research was undertaken to investigate the responsible mechanisms. Oxidative stress has been associated with a considerable number of pathologies and thus it was considered a logical area to investigate (McCord, 2000). Furthermore, the pattern of hypoxia and re-oxygenation in OSA-related IH is recognised to be similar to that occurring during ischaemia-reperfusion injury (IRI) where oxidative stress is shown to play a major role in the related tissue injury. Therefore, there has been a considerable volume of animal research and recently a number of human studies to investigate whether IH elicits oxidative stress and whether this could mediate the development of cardiovascular disease in OSA.

(Gerschman et al., 1954) first presented the theory that reduced forms of O₂ called free radicals could be toxic to the human body. (McCord and Fridovic, 1969) later discovered the antioxidant enzyme superoxide dismutase (SOD) thus demonstrating the importance of free radicals in biological systems. Free radicals is an umbrella term for both reactive oxygen species (ROS, e.g. superoxide anion) and reactive nitrogen species (RNS, e.g. nitric oxide) which are molecules or fragments of molecules that have one or more unpaired electron, which makes

them so reactive (Halliwell and Gutteridge, 1999). Sources of free radical generation include the mitochondrial electron transport chain, peroxisomes and enzymes such as, NAD(P)H oxidase and xanthine oxidase (Valko et al., 2007).

To prevent biological damage caused by the generation of excess free radicals, endogenous and exogenous antioxidants are required to quench and buffer free radicals. Enzymatic antioxidant defences include, superoxide dismutase, glutathione peroxidase and catalase. Non-enzymatic antioxidants include ascorbic acid (Vitamin C), alpha-tocopherol (Vitamin E), glutathione, carotenoids, and flavonoids (Valko et al., 2007). During pathological conditions such as, OSA and diabetes or during periods of excess stress (e.g. severe exercise) an overproduction of free radicals and/or insufficient antioxidants can lead to oxidative stress. Oxidative stress is a process where free radicals can damage cellular proteins (e.g. protein carbonylation), lipids (e.g. lipid peroxidation), carbohydrate structures and DNA, all of which can inhibit cellular function. Oxidative stress has been associated with a considerable number of pathologies such as all inflammatory diseases, diabetes, ischaemic diseases (stroke, myocardial infarction) and Alzheimer's disease (McCord, 2000).

Because OSA patients are often obese and have coexisting medical disorders such as type 2 diabetes mellitus (T2DM) studies have normally utilised CPAP treatment to assess whether there is a casual relationship between OSA and elevated oxidative stress. Studies measuring biomarkers of oxidative stress in the

plasma have reported elevated levels of lipid (Barcelo et al., 2000, Lavie et al., 2004) and protein oxidation (Mancuso et al., 2012) in OSA patients vs. controls and demonstrated an attenuation following CPAP treatment. Studies have also demonstrated that intracellular ROS production in leukocytes (Dyugovskaya et al., 2002, Schulz et al., 2000) and oxidative stress in venous endothelial cells is elevated in OSA patients and restored by CPAP treatment (Jelic et al., 2010, Jelic et al., 2008). Finally, plasma antioxidant levels have been shown to be attenuated in OSA patients which would indicate a chronic overproduction of free radicals and CPAP treatment was also able to reverse this (Barcelo et al., 2006, Mancuso et al., 2012).

1.3.2 IH-induced oxidative stress

Elevations in biomarkers of oxidative stress in the plasma (Christou et al., 2009) and urine (Yamauchi et al., 2005) of OSA patients have been shown to be correlated to hypoxic indices such as, the lowest and mean nadir in S_aO_2 following apnoeas and the frequency of these O_2 de-saturation events. Furthermore, recent studies have reported increased levels of biomarkers of oxidative stress in the plasma of healthy humans and OSA patients following acute exposure to intermittent reductions in P_{IO_2} (Pialoux et al., 2009, Lee et al., 2009). The first of these two studies reported an increase in DNA and lipid oxidation in healthy humans after a six hour cycle of two minutes hypoxia followed by two minutes euoxia (Pialoux et al., 2009). In the later study even much shorter durations of IH

(twelve cycles of four minutes hypoxia and four minutes euoxia) were capable of inducing DNA oxidation in OSA patients but not in healthy individuals (Lee et al., 2009).

Similar to the aforementioned findings in OSA patients and in healthy humans exposed to acute IH, animals exposed to chronic IH demonstrate greater levels of lipid and protein oxidation (Xu et al., 2004, Zhan et al., 2005) and develop an impaired antioxidant capacity (Chen et al., 2005). Furthermore, in one study an increase in lipid oxidation was significantly correlated to elevations in blood pressure and left ventricular dysfunction (Chen et al., 2005). There has long been much debate over whether free radicals and oxidative stress are causative in pathological conditions such as the development of atherosclerosis, or whether they are simply a consequence of the pathology. Indeed, although *in vitro* evidence for oxidative stress mediating atherosclerosis is strong, large scale clinical trials of antioxidants have had minimal to no success in attenuating atherosclerosis or cardiovascular events mediated by atherosclerosis (Stocker and Keaney, 2004, Steinberg, 2009). In contrast, a number of studies have presented strong evidence suggesting IH-induced oxidative stress is causative in the development of cardiovascular disease rather than a consequence (Burckhardt et al., 2008, Troncoso Brindeiro et al., 2007, Peng et al., 2006). Firstly, IH-induced oxidative stress would appear to mediate systemic hypertension in chronic IH rodents as studies have shown antioxidant treatment attenuates the rise in systemic blood pressure and elevated levels of circulating endothelin-1 (Troncoso Brindeiro et al.,

2007) and plasma noradrenalin (Peng et al., 2006). Furthermore, treatment with the antioxidant green tea polyphenol in chronic IH animals was shown to attenuate the elevation in levels of lipid oxidation in the brain and improve cognitive function (Burckhardt et al., 2008).

1.3.3 Mechanisms of IH-induced oxidative stress

Although it is now well established that exposure to IH can evoke oxidative stress, the responsible mechanism(s) for increased free radical production during IH remain incompletely understood. As discussed earlier there are numerous sources for free radical production (e.g. mitochondria, enzymes etc) that have been identified in a range of different cells (endothelial and leukocytes) and almost all of these have been suggested as responsible sources of the overproduction of free radicals during IH (Lavie, 2003). As mentioned earlier, the cycle of hypoxia and re-oxygenation during IH closely resembles the changing O₂ tensions occurring in IRI. IRI is associated with organ transplantation, strokes and myocardial infarctions whereby the blood supply to a specific tissue/organ is blocked for a lengthy duration (ischaemia) causing severe hypoxia. Upon reperfusion and subsequent re-oxygenation there is a substantial generation of free radicals that cause significant oxidative stress and impair tissue/organ function. Although there are striking differences between the pattern of hypoxia and re-oxygenation in OSA-induced IH and IRI (e.g. duration, hypoxic severity and number of hypoxia/re-oxygenation cycles) the best predictions for the responsible mechanisms of free

radical generation in IH are based primarily on the findings from research investigating IRI.

Figure 1.2 illustrates the proposed primary mechanisms responsible for increased ROS production during a cycle of hypoxia and re-oxygenation in IRI. Firstly, an increase in ROS generation may occur in the mitochondria during hypoxia. Secondly, the enzyme xanthine oxidase may increase ROS generation during re-oxygenation and thirdly a subsequent inflammatory response may increase ROS production via NAD(P)H oxidase during late reperfusion and for a lengthy time thereafter.

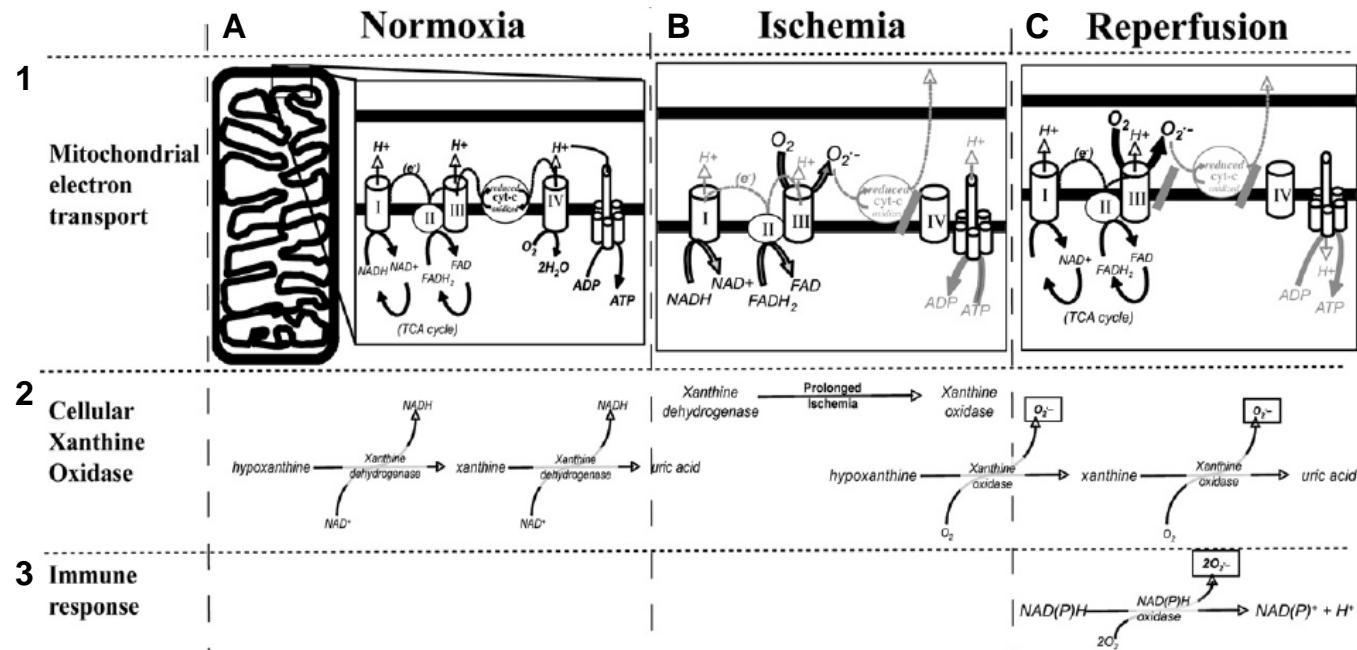


Figure 1.2: A schematic adapted from Raedschelders et al. (2012) showing the responsible mechanisms for increased ROS generation during IRI that may also be responsible for elevated ROS during OSA-related IH. **A1:** Normal oxidative phosphorylation functioning; **B1:** Inhibition of electron flux beyond complex III and a reduced NADH:NAD⁺ ratio due to impaired TCA cycle function during ischemia/hypoxia depletes the mitochondrial cytochrome C pool and subsequently causes a small increase in the generation of superoxide ($O_2^{\bullet-}$); **C1:** Restored TCA cycle during reperfusion with a continued inhibition of electron flux beyond complex III augments $O_2^{\bullet-}$ generation; **A2:** Xanthine dehydrogenase activity under euoxic conditions during normal perfusion; **B2:** Prolonged ischemia/hypoxia converts xanthine dehydrogenase to xanthine oxidase and increases hypoxanthine and xanthine concentrations; **C2:** Xanthine oxidase couples hypoxanthine and xanthine with O_2 to generate $O_2^{\bullet-}$; **C3:** Stimulated immune cells during reperfusion generate $O_2^{\bullet-}$ via NAD(P)H oxidase subsequent to xanthine oxidase-induced ROS production.

Free radical formation during ischaemia (hypoxia): Tissue injury following IRI is commonly regarded as resulting predominantly from an overproduction of ROS during reperfusion (i.e. re-oxygenation) but there are also reports of significant elevation in the generation of ROS during ischaemia (Becker et al., 1999, VandenHoek et al., 1997, Zweier et al., 1987, Zweier et al., 1989). During non pathological conditions, intracellular superoxide is produced primarily by the mitochondria during oxidative phosphorylation when electrons leak prematurely to O₂ molecules (Valko et al., 2007). Despite hypoxia presumably reducing rates of oxidative phosphorylation, mitochondrial generation of ROS increases due to an increased “leakage” of electrons from complex III of the electron transport chain (Raedschelders et al., 2012, Valko et al., 2007).

Free radical formation during early reperfusion (re-oxygenation): The enzyme xanthine oxidase was identified during early research into IRI as a likely source of superoxide generation during reperfusion (McCord, 1985, Schoutsen et al., 1983). Xanthine oxidase and xanthine dehydrogenase are inter-convertible forms of the same enzyme, xanthine oxidoreductase. Unlike xanthine dehydrogenase that catalyses the oxidation of hypoxanthine to xanthine and subsequently to uric acid by coupling the reaction with NAD⁺, xanthine oxidase does so by coupling the reaction with O₂ and produces superoxide rather than NADH. During ischaemia impairment in cellular regulatory mechanisms can mediate the conversion of xanthine dehydrogenase to xanthine oxidase (Engerson et al., 1987, McKelvey et al., 1988). Furthermore, reduced oxidative phosphorylation during hypoxia results

in an increased cellular breakdown of energy rich metabolites and a subsequent increase in the availability of hypoxanthine to be catalysed by xanthine oxidase (i.e. adenosine triphosphate (ATP) to adenosine monophosphate (AMP) to adenosine to inosine and finally hypoxanthine) (Thompsongorman and Zweier, 1990, Zweier et al., 1994).

The contribution that xanthine oxidase activity may have on oxidative stress and tissue damage in IRI has been investigated using the xanthine oxidase inhibitor allopurinol. Allopurinol preserved antioxidant capacity in myocardial tissue of dogs (Chambers et al., 1985, Peterson et al., 1985), improved myocardial function and coronary flow in rats (Thompsongorman and Zweier, 1990) and reduced tissue damage following open heart surgery in humans (Gimpel et al., 1995). Allopurinol has also been given to OSA patients and animals exposed to chronic IH to determine whether xanthine oxidase activity also mediates the elevation in oxidative stress in OSA-induced IH. Allopurinol treatment lowered oxidative stress and improved endothelial function (assessed by flow mediated dilatation) in OSA patients (El Solh et al., 2006) and reduced oxidative stress and cardiac tissue damage in rodents exposed to chronic IH (Williams et al., 2010).

Free radical formation during a delayed inflammatory response (late reperfusion/re-oxygenation): Tissue damage during reperfusion evokes a large inflammatory response and elevated levels of ROS generation were seen for several hours post-reperfusion (Bolli et al., 1989, Bolli et al., 1988). Increased

activity of the enzyme NAD(P)H oxidase found predominantly in immune cells may contribute heavily to the delayed accumulation of ROS (Raedschelders et al., 2012). The comparably brief duration of hypoxic episodes in IH that occur in OSA would not be expected to elicit the same magnitude of local inflammation as IRI. However, OSA is a chronic condition and involves several hundred re-oxygenation phases per night which affects the entire body rather than just a single organ. Indeed, evidence exists to suggest a heightened inflammatory state in OSA patients (Lavie and Lavie, 2009).

1.4 IH-induced oxidative stress during the postprandial state

Following consumption of a meal the body is in a postprandial state whereby digestion of food is occurring in the gastrointestinal tract and subsequently small molecules move into the blood and are then involved in many metabolic processes. Although the term postprandial is used in many contexts, it is most commonly used when describing the relatively rapid rise and short duration of elevated blood glucose levels following a meal (i.e. postprandial hyperglycaemia) and also the more gradual and sustained elevation in circulating lipids (i.e. postprandial hyperlipidaemia). Recent evidence suggests postprandial hyperglycaemia and postprandial hyperlipidaemia could affect the magnitude of oxidative stress during IH. This new evidence may be relevant to all OSA patients especially those with coexisting T2DM who consume meals high in glucose and lipid prior to sleep.

T2DM is a metabolic condition characterised by elevated blood glucose levels due to insulin resistance that can progress to an additional deficiency in insulin production. There are many risk factors for the development of T2DM such as, increased age and a lack of physical exercise but by far the greatest risk factor is excess weight. Thus, because excess weight is also the strongest predictor of OSA over half of patients with T2DM suffer from OSA (Einhorn et al., 2007), with a coexistence as high as 86% in obese patients with T2DM (Foster et al., 2009). Although T2DM is most commonly associated with elevated fasting blood glucose levels, insulin resistance also means they experience substantial augmentations in postprandial hyperglycaemia and hyperlipidaemia (Ceriello et al., 2002). Oxidative stress is believed to be heavily involved in the pathogenesis of vascular complications of T2DM primarily as a consequence of hyperglycaemia (Son, 2012, Brownlee, 2001). Indeed, it has been shown that postprandial hyperglycaemia causes increased oxidative stress in T2DM patients (Ceriello et al., 2002). Furthermore, postprandial hyperlipidaemia also causes a transient increase in oxidative stress in healthy individuals which is greatly augmented in T2DM and thus may also play a role in mediating the health complications of T2DM (Ceriello et al., 2002).

1.4.1 Simultaneous exposure to IH and postprandial hyperglycaemia

Following consumption of an evening meal, blood glucose levels in insulin-sensitive individuals will normally have returned to pre-meal levels prior to sleep. However, because T2DM patients experience an augmentation in the magnitude

and also duration of postprandial hyperglycaemia blood glucose levels are likely to remain elevated during the initial hours of sleep. Therefore, it is likely that OSA patients with coexisting T2DM will experience IH simultaneously with postprandial hyperglycaemia.

We have become aware of a line of evidence that may suggest that simultaneous exposure to these two stimuli could be extremely detrimental to health. As discussed previously (see: *mechanisms for IH-induced oxidative stress*) the pattern of cellular hypoxia and re-oxygenation during IH mimics that occurring in IRI and it is widely considered that the mechanisms responsible for the overproduction of ROS during IRI are the same as those occurring during IH (Lavie, 2003, Pack and Gislason, 2009). Interestingly, transient hyperglycaemia has been shown to have a negative effect on morbidity and mortality in humans following events characterised by IRI such as, stroke and myocardial infarctions (Doenst et al., 2005, Ouattara et al., 2005, Schmeltz et al., 2007). Furthermore, experimental studies with rodents have shown that transient hyperglycaemia during IRI increased liver (Behrends et al., 2010), kidney (Hirose et al., 2008) and cerebral tissue damage (Bemeur et al., 2007, Tsuruta et al., 2010). During acute modest elevations in ROS production antioxidant defences in healthy humans and animals are capable of inhibiting or greatly attenuating oxidative stress. It is plausible that the greater rates of morbidity and mortality in humans following IRI with simultaneous hyperglycaemia (Doenst et al., 2005, Ouattara et al., 2005, Schmeltz et al., 2007) was a result of an additive effect of ROS production from hyperglycaemia and IRI independently, which

overwhelmed antioxidant defences causing a substantially greater degree of oxidative stress. Indeed, all of the experimental animal studies investigating simultaneous hyperglycaemia and IRI showed greater levels of oxidative stress (Behrends et al., 2010), (Hirose et al., 2008, Bemeur et al., 2007, Tsuruta et al., 2010). However, evidence from two of these animal studies strongly suggest that increased oxidative stress and tissue damage occurring during IRI under hyperglycaemic conditions and thus possibly IH are not simply a result of a cumulative increase in ROS production from the two ROS-producing stimuli (Hirose et al., 2008, Tsuruta et al., 2010). Rather, it advocates that hyperglycaemia can interact with and increase the activity of a pathway(s) responsible for ROS production during IRI. Figure 1.3 is taken from (Tsuruta et al., 2010) and it shows how 30 minutes of hyperglycaemia alone prior to ischaemia caused no increase in ROS generation but significantly increased ROS generation during ischaemia and especially following re-perfusion. Furthermore, (Hirose et al., 2008) showed that 24 hours after renal IRI, oxidative stress and renal necrosis were significantly greater when mice were exposed to hyperglycaemia simultaneously with IRI but that hyperglycaemia initiated two hours after reperfusion had no effect on oxidative stress levels.

Considering the high prevalence of coexisting OSA and T2DM and the aforementioned line of evidence demonstrating a synergistic interaction between IRI and hyperglycaemia, research is required to examine whether the same synergy is present with simultaneous exposure to IH and hyperglycaemia.

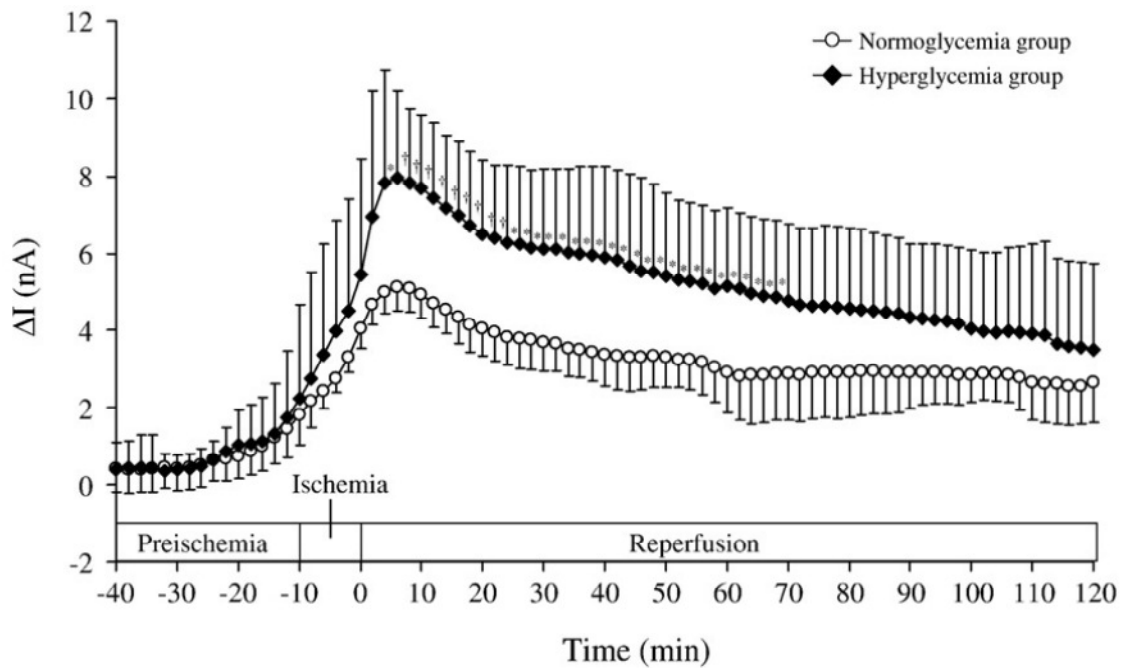


Figure 1.3: Reactive oxygen species production (ΔI (nA)) during cerebral ischaemia-reperfusion injury (IRI). Generation of ROS under hyperglycaemic (closed symbols) or euglycaemic conditions (open symbols). Note a lack of difference between ROS production between hyperglycaemia and euglycaemia during pre-ischaemia, but greater elevations under hyperglycaemia during ischaemia and reperfusion (Tsuruta et al., 2010).

1.4.2 Simultaneous exposure to IH and postprandial hyperlipidaemia

Postprandial hyperlipidaemia occurs more gradually than postprandial hyperglycaemia and thus circulating lipid levels remain above fasting levels for many hours longer than postprandial hyperglycaemia. Therefore, OSA patients who consume a high lipid evening meal within a few hours of sleeping may experience simultaneous exposure to postprandial hyperlipidaemia and IH. In OSA patients with coexisting T2DM, this simultaneous exposure may even occur when

meals are consumed much earlier in the evening because T2DM demonstrate augmented magnitude and duration of postprandial hyperlipidaemia (Ceriello et al., 2002).

In healthy humans, postprandial hyperlipidaemia causes a modest transient elevation in oxidative stress but in T2DM patients this response is augmented (Ceriello et al., 2002). It is currently unknown why oxidative stress is greater in T2DM patients but it is likely to be in part a result of the exaggerated magnitude of postprandial hyperlipidaemia and an already lower antioxidant capacity (Baynes, 1991). We are not aware of any existing research that has examined the effects of simultaneous exposure to hyperlipidaemia and IH at any biological level. Therefore, it remains to be determined whether combined exposure to IH and postprandial hyperlipidaemia overwhelms the antioxidant defence system causing substantially greater oxidative stress than individual exposure to each stressor.

In summary, OSA patients who consume evening meals shortly before sleep may experience postprandial hyperglycaemia and hyperlipidaemia simultaneously with IH. Postprandial hyperglycaemia and hyperlipidaemia are both capable of causing increased ROS generation but it remains to be determined what effect simultaneous exposure to these stimuli with IH causes. Acute hyperglycaemia independently does not increase ROS production in rodents but greatly augments ROS production during IRI and thus may also have a synergistic effect during IH.

This could have implications for all OSA patients but especially the high prevalence of those with coexisting T2DM.

1.5 IH-mediated respiratory plasticity

1.5.1 General

The term neuroplasticity has no clear scientific definition and encompasses in its entirety any physiological adaptation of the central nervous system (from molecular to a change in physical structure) that can occur in response to a large range of different stimuli such as environment or behaviour. Neuroplasticity has long been recognised as being fundamental for learning and memory. In contrast the neural system responsible for respiratory control has conventionally been considered 'rigid' even during early development where other neural systems are known to demonstrate their greatest capacity for neuroplasticity. This traditional belief has since been challenged and substantial evidence now exists to indicate the respiratory system is capable of considerable neuroplasticity (Mitchell and Johnson, 2003). The respiratory motor control system regulates the essential process of all mammals' life: breathing. It is therefore logical that neuroplasticity is an integral component of the respiratory system that guarantees precise control of breathing in order to maintain blood gas homeostasis during changing conditions. These conditions may be physiological, such as ageing, pregnancy, ascent to altitude, or pathophysiological conditions such as respiratory infections and

lung/neural disease. Most of the changing conditions experienced throughout life that evoke respiratory plasticity are long lasting and develop gradually, such as weight gain and ageing. Their respective development of respiratory plasticity is also relatively gradual. However, respiratory plasticity can also develop more quickly in response to conditional changes, such as respiratory infection and ascent to altitude.

Animals and humans naive to prior hypoxic exposure develop two forms of respiratory plasticity that appear unique to acute IH and are rapid in their onset. These are, respiratory long-term facilitation (LTF) and progressive augmentation of the hypoxic ventilatory response (HVR). HVR is the term given to the elevation in ventilation in response to a lowered Pa_{O_2} . Respiratory LTF is a persistent and progressive augmentation in respiratory motor output that is initiated during euoxic episodes that intersperse hypoxic exposures and is sustained for minutes to hours beyond cessation of IH (Mitchell and Johnson, 2003). Progressive augmentation of the HVR is referred to as a gradual increase in the magnitude of HVR during sequential hypoxic episodes of identical intensity and duration that can be sustained for over one hour post acute IH (Mateika et al., 2004, Powell et al., 1998).

Before discussing these phenomena in greater detail it is necessary to provide some definitions for the various forms of neuroplasticity and indicate where

respiratory LTF and progressive augmentation of the HVR are positioned. However, because of the relative infancy of research into neuroplasticity of the respiratory motor control system, a clear framework of respiratory plasticity remains to be universally accepted. (Mitchell and Johnson, 2003) attempted to establish a clear set of definitions of plasticity and other related neural properties that affect respiratory motor control. Figure 1.4 is adapted from (Mitchell and Johnson, 2003) to illustrate the different forms of respiratory plasticity that are specific to IH. Respiratory modulation (panel A) is a neurochemically induced alteration in respiratory neural network function that is only active during the presence of the neurochemical e.g. serotonergic modulation of respiratory neurones during hypoxic exposure but is absent as soon as euoxia is resumed. Respiratory metamodulation (panel B) is an enhancement or reduction of the neurochemically induced alteration in respiratory neural network, e.g. progressive augmentation of the HVR during IH exposure. Respiratory Plasticity (panel C) is a sustained alteration in respiratory neural network function that is sustained beyond cessation of the stimulus, e.g. respiratory LTF. Respiratory metaplasticity (panel D) is a change in the magnitude of respiratory plasticity induced by prior or concurrent experience, e.g. the magnitude of respiratory LTF is enhanced in humans with OSA (Lee et al., 2009) or animals exposed to chronic IH (Peng et al., 2003).

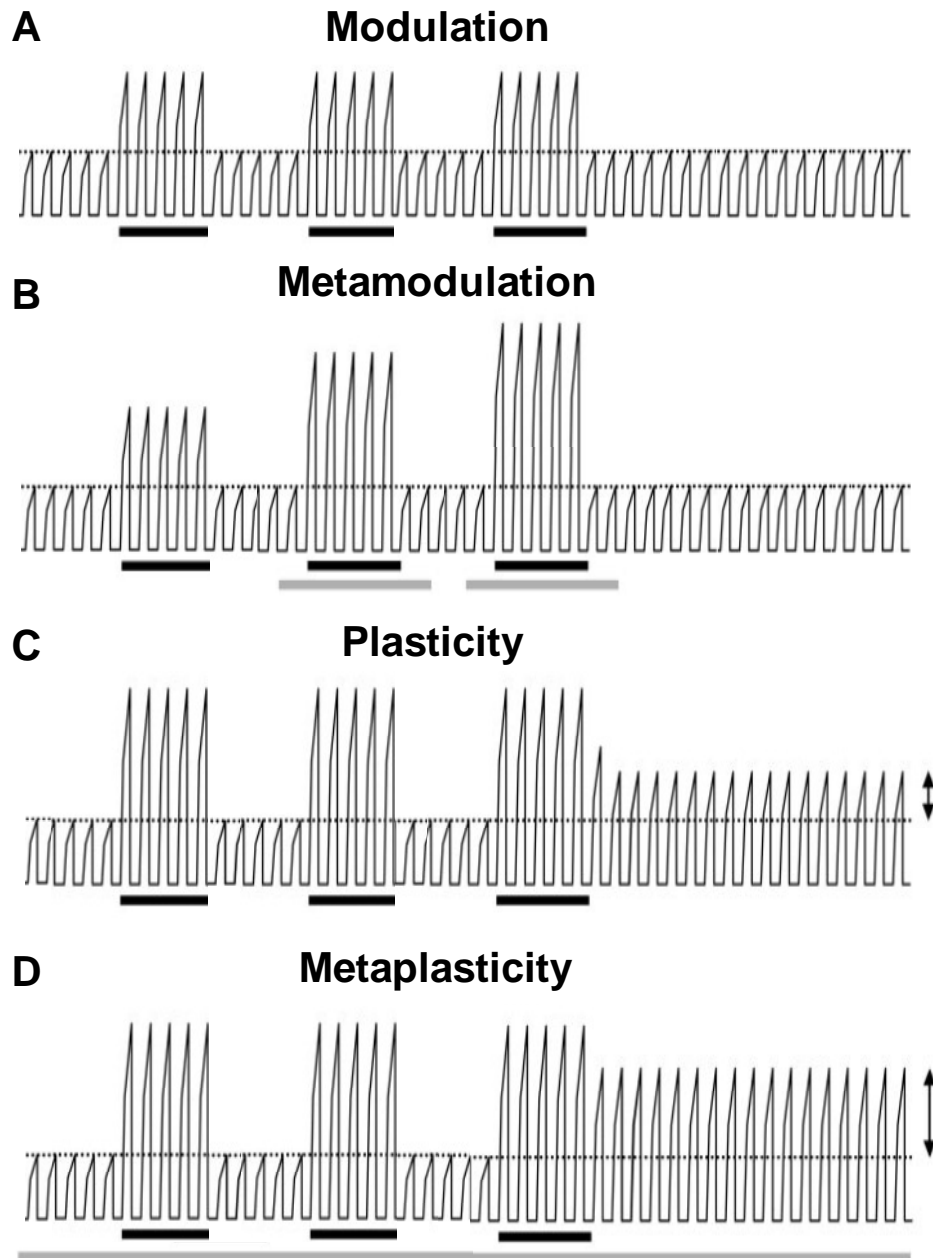


Figure 1.4: Schematic adapted from Mitchell and Johnson (2003) illustrating IH-induced respiratory modulation and respiratory plasticity. The tracing represents respiratory motor output with solid black bars represent hypoxic exposure and solid grey bars indicate previous exposure to IH.

Although progressive augmentation of the HVR clearly fits well into the metamodulation category described by (Mitchell and Johnson, 2003) it may be an extension of respiratory LTF as discussed later (see: *Is progressive augmentation of the HVR the same phenomenon as respiratory LTF and what are the responsible mechanism(s)?*). It is also commonly defined as a form of respiratory plasticity in the literature (Mateika and Narwani, 2009) and for simplicity this thesis will conform with the current literature and progressive augmentation of the HVR will be discussed as a form of respiratory plasticity.

1.5.2 Why investigate IH-mediated respiratory plasticity?

In the 32 years since (Millhorn et al., 1980a) first reported respiratory LTF in cats following intermittent stimulation of the carotid sinus nerve a large volume of research has been undertaken to investigate the mechanisms responsible for this phenomenon. Indeed, a recent review by (Mateika and Sandhu, 2011) reported that there are more than 70 published animal studies to date to have specifically investigated respiratory LTF and numerous human studies. Progressive augmentation of the HVR has received far less attention than respiratory LTF but there still exists a considerable number of studies that have increased our understanding of this phenomenon (Mateika and Narwani, 2009). In general the beneficial significance of respiratory plasticity is to maintain normal arterial blood gas levels and it has been well described for a number of conditions. For example, elevated ventilation induced by acclimatisation to chronic altitude exposure partly balances the reduction in atmospheric O₂ and alterations in respiratory output

during pregnancy overcome the mechanical limitations. However, our understanding of the possible benefits that respiratory plasticity following IH may have are less well understood.

Could OSA-induced IH evoke respiratory plasticity and thus attenuate upper airway collapse?

OSA patients experience IH on a nightly basis which theoretically could elicit respiratory plasticity as is demonstrated in animals and humans following exposure to intermittent reductions in P_{IO_2} (Mateika and Sandhu, 2011). If OSA does elicit respiratory plasticity it must then be considered whether respiratory LTF and/or progressive augmentation of the HVR are beneficial or detrimental to upper airway stability in OSA patients (Mateika and Narwani, 2009). Based on our current understanding of the function of respiratory plasticity in other settings (e.g. acclimatisation to altitude) it seems logical that respiratory plasticity in OSA patients should serve to stabilise breathing, preventing apnoeas and thus maintaining blood gas homeostasis (Mahamed and Mitchell, 2007). In OSA upper airway collapse occurs during sleep but not wakefulness due to a reduction in neural activity to the pharyngeal dilator muscles during sleep. Therefore, LTF of neural activity to upper airway dilator muscles would be expected to increase muscle tone and thus attenuate upper airway collapse. Upon arousal and restoration of the upper airway a period of hyperventilation also occurs which can evoke hypocapnia causing a subsequent reduction in respiratory drive. It is well known that upper airway dilator muscle activity parallels the level of ventilation and thus the upper airway is prone

to collapse during nadirs in ventilation. Furthermore, if PaCO_2 levels are driven below the apnoeic threshold this greatly increases the risk of a central apnoea (Mateika and Narwani, 2009) which can then be followed by collapse of the upper airway and thus an obstructive apnoea (Badr, 1996, Badr et al., 1997, Hudgel et al., 1987, Onal et al., 1986). By maintaining respiratory drive during these periods of hypocapnia ventilatory LTF may prevent further obstructive events and thus attenuate OSA severity. In contrast, development of progressive augmentation of the HVR might elicit an unsuitably exaggerated response to apnoea-induced hypoxia (i.e. increased respiratory control system loop gain), such that considerably greater hypocapnia is elicited. This will lead to a reduction in ventilatory drive and a parallel reduction in upper airway dilator muscle activity increasing the chance of upper airway collapse. Therefore, it is believed by many that progressive augmentation of the HVR could destabilise the upper airway in OSA patients.

An interesting finding is that 78% of OSA patients demonstrate long periods of stable breathing characterised by the absence of apnoeas (Younes, 2003). If these long periods of stable breathing are ascribed to IH evoked respiratory LTF it should also be expected that apnoea severity should lessen over the course of the night. However, a number of clinical studies have reported that the apnoea hypopnoea index (AHI) (Fanfulla et al., 1997, Sforza et al., 1998) and duration of apnoeas (Sforza et al., 1998, Charbonneau et al., 1994) increases rather than decreases

throughout the night. Furthermore, because chronic exposure to IH enhances respiratory LTF (i.e. metaplasticity is present) (Ling et al., 2001), it would be expected that OSA severity would decrease over time in OSA patients but in contrast it worsens. However, it remains to be determined whether this is due to risk factors of OSA such as, obesity increasing over time (Chamberlin and Ling, 2011). In summary, these studies suggest that respiratory LTF is not expressed in OSA patients, or, if it is expressed, it is incapable of improving breathing stability.

Potential benefits of artificially inducing or enhancing respiratory plasticity

The aforementioned evidence clearly argues against any naturally developed respiratory LTF promoting breathing stability in OSA. However, it does not preclude the potential benefit to breathing stability of voluntarily inducing respiratory LTF prior to sleep, or enhancing the natural ability of nocturnal IH to induce respiratory LTF. A clearer understanding of the mechanisms responsible for respiratory LTF may enable the development of new therapeutic strategies to artificially induce respiratory LTF or enhance the inherent capacity for IH-induced respiratory LTF. Furthermore, an understanding of the different mechanisms (if they are different) between the various forms of respiratory plasticity (e.g. LTF of ventilation, progressive augmentation of the HVR, and upper airway dilator muscles) would allow therapeutic strategies to be tailored to induce only the forms of respiratory plasticity that are deemed beneficial to breathing stability.

Other potential benefits of respiratory plasticity

Although research into respiratory plasticity has primarily been performed because of its perceived potential to promote breathing stability and mitigate OSA, its benefits may not be restricted to OSA. Indeed, a greater understanding of respiratory plasticity may guide the development of therapeutic interventions for the treatment of other conditions associated with respiratory insufficiency, such as chronic lung disease, sudden infant death syndrome, congenital alveolar hypoventilation syndrome, and neuromuscular injury (Mitchell and Johnson, 2003). Fascinating data from animal research is emerging to suggest that chronic exposure to IH may be an effective means of restoring breathing capacity following chronic spinal injury (Dale-Nagle et al., 2010, Lovett-Barr et al., 2012). Even if research aimed at increasing our understanding of respiratory plasticity does not lead to the development of therapeutic strategies for respiratory related illness, our enhanced knowledge of neuroplasticity derived from these studies may be relevant to other neural systems. Indeed, our current understanding of IH-induced respiratory plasticity has already begun to impact on the development of treatments for non respiratory related neuronal conditions (Trumbower et al., 2012). (Trumbower et al., 2012) recently demonstrated that acute IH evokes sustained increases in somatic motor output in patients with chronic incomplete spinal cord injury.

For the remainder of this chapter a detailed discussion regarding the findings from animal studies investigating respiratory LTF and the more limited research

performed in humans is given. Finally, a shorter discussion is presented regarding progressive augmentation of the HVR where findings from animal and human studies are combined.

1.5.3 Respiratory LTF in animals

Respiratory LTF was first described by (Millhorn et al., 1980a) although the term *respiratory LTF* was established later. Anaesthetised, paralysed, vagotomised and mechanically ventilated cats were either exposed to five 2-minute episodes of carotid sinus nerve stimulation with five-minute intervals, or the same duration/interval of rapid warming of a previously cooled carotid body (CB) in order to induce the same intermittent firing pattern of the carotid sinus nerve. Phrenic activity increased above baseline following both intermittent carotid sinus nerve stimulation and intermittent carotid activation and remained above baseline for more than 90 minutes. This sustained elevation in phrenic activity was termed phrenic LTF and has since been replicated in cats (Millhorn et al., 1980b, Fregosi and Mitchell, 1994) and rats (Hayashi et al., 1993, Ling et al., 1997). Although intermittent carotid sinus nerve stimulation was used to induce phrenic LTF in these early studies investigating respiratory LTF, it was soon demonstrated that exposure to acute IH was also capable of inducing phrenic LTF (Hayashi et al., 1993, Bach and Mitchell, 1996).

Respiratory LTF in different respiratory motor nerves

In addition to phrenic LTF a substantial number of studies have since reported acute IH-induced respiratory LTF in various other respiratory motor pools in a range of different species (Mateika and Sandhu, 2011, Baker-Herman and Strey, 2011). These include LTF of hypoglossal (Bach and Mitchell, 1996), glossopharyngeal (Cao et al., 2010) and intercostal motor nerves (Fregosi and Mitchell, 1994). However, in comparison to phrenic LTF relatively little research has been performed on hypoglossal LTF and considerably less again on LTF of glossopharyngeal and intercostal motor nerves. As a result far less is understood about the mechanisms of LTF of these motor nerves compared with the phrenic nerve. Although they appear to be fundamentally very similar (i.e. require serotonin receptor activation and require ROS) emerging evidence suggests there may be distinct mechanistic differences (e.g. variations in the magnitude of LTF and hypoxic pattern sensitivity) (Baker-Herman and Strey, 2011).

Ventilatory LTF in non-anaesthetised animals

As discussed previously, the beneficial significance of respiratory plasticity is to maintain normal arterial blood gas levels during altered environmental or physiological conditions. Therefore, for phrenic LTF to have a significant physiological impact it must affect ventilatory output under non-anaesthetised conditions (e.g. awake or sleeping). Indeed, ventilatory LTF following acute IH has been reported in awake goats (Turner and Mitchell, 1997), ducks (Mitchell et al., 2001b), rats (Olson et al., 2001, McGuire et al., 2002), mice (Kline et al., 2002,

Terada et al., 2008), dogs (Cao et al., 1992) and also in sleeping rats and mice (Nakamura et al., 2010, Terada et al., 2008). It would thus appear that the extensive research in anaesthetised animals focusing on respiratory motor neurone LTF is of potential clinical importance, as ventilatory LTF can clearly manifest.

Requirement for CO₂

It was realised early on by investigators that reduced chemoreceptor feedback by hypoxia-induced hypocapnia could restrain the expression of respiratory LTF. Therefore, the majority of studies investigating respiratory LTF in anaesthetised animals would first determine the baseline PaCO₂ of the apnoeic threshold. PaCO₂ levels were then raised slightly above the apnoeic threshold and maintained throughout the study. When PaCO₂ was maintained 2-3 mmHg above the apnoeic threshold, anaesthetised rodents demonstrate a significant increase in phrenic amplitude of 63% (Bach and Mitchell, 1996), 37% (Kinkead and Mitchell, 1999), 78% (Baker and Mitchell, 2000), and 57% (Fuller et al., 2001) above baseline at 60 minutes following acute IH. Furthermore, the restraining effect of hypoxia-induced hypocapnia on ventilatory LTF is clearly evident in non-anaesthetised rats as an increase in ventilation of only 20% was evident 60 minutes post acute IH but increased to 57% when supplemental CO₂ was subsequently given to return PaCO₂ to baseline levels (Olson et al., 2001). In addition, exposure to acute IH with 10% O₂ induced ventilatory LTF in rats but exposure to acute IH with 8% O₂ did not

(McGuire et al., 2002). The authors suggested that the inhibition of ventilatory LTF during the 8% O₂ exposure was due to a greater HVR that produced a greater degree of hypocapnia.

Respiratory LTF in animals is pattern sensitive

Although there is overwhelming evidence that respiratory LTF can be evoked by acute IH in various anaesthetised and conscious animal species (Mateika and Sandhu, 2011) acute CH does not appear to be a stimulus capable of eliciting respiratory LTF (Turner and Mitchell, 1997, Dwinell et al., 1997, Baker and Mitchell, 2000, Mitchell et al., 2001b, Tadjalli et al., 2007). Furthermore, respiratory LTF following acute IH (Bach and Mitchell, 1996) has been shown to be mediated by a serotonin-dependant spinal cord mechanism (see the next section for details) and only intermittent but not continuous application of serotonin or serotonin receptor (5-HT₂) agonists to the spinal cord evokes phrenic (Lovett-Barr et al., 2006, MacFarlane and Mitchell, 2009) and hypoglossal LTF (Bocchiaro and Feldman, 2004).

Mechanisms of respiratory LTF

Because of the potential to use respiratory LTF as a treatment for OSA and respiratory conditions associated with spinal cord injury, there exists a considerable body of research specifically investigating the mechanisms responsible for respiratory LTF. The majority of this research has focused on phrenic LTF and there is overwhelming support for a serotonin-dependent

mechanism initiating and maintaining it. Upon first reporting the existence of phrenic LTF following intermittent carotid sinus nerve stimulation (Millhorn et al., 1980b) demonstrated that the mechanism involved activation of serotonin receptors because systemic pre-treatment with a serotonin receptor antagonist (methysergide) inhibited its development. Furthermore, (Bach and Mitchell, 1996) later showed that systemic methysergide administration also prevented phrenic LTF evoked by exposure to acute IH. Subsequent studies have since progressed our understanding of this serotonin-dependant mechanism for respiratory LTF and a well accepted model has been proposed (Mahamed and Mitchell, 2007, MacFarlane et al., 2008). Figure 1.5 is adapted from Mahamed & Mitchell (2007) demonstrating the proposed serotonin-dependant mechanism for respiratory LTF. Phrenic LTF requires spinal serotonin release from medullary raphe serotonergic neurons during hypoxic exposure (Baker-Herman and Mitchell, 2002) and subsequent activation of serotonin receptors (5-HT₂) on respiratory motoneurons in the spinal cord (Kinkead and Mitchell, 1999, Fuller et al., 2001). This in turn initiates spinal protein synthesis of brain-derived neurotrophic factor (BDNF) that subsequently activates its endogenous high affinity receptor tyrosine kinase (TrkB) that maintains phrenic LTF (Baker-Herman et al., 2004). Far less research examining the mechanisms for LTF in other respiratory motor pools has been performed (Baker-Herman and Strey, 2011). However, studies have shown that intermittent application of 5-HT₂ agonists on or near the respiratory motor pool induce hypoglossal LTF (Bocchiaro and Feldman, 2004). Furthermore, both hypoglossal and glossopharyngeal LTF are 5-HT₂ receptor dependant (Fuller et al.,

2001, Cao et al., 2010), suggesting LTF of all inspiratory motor pools may be serotonin dependant.

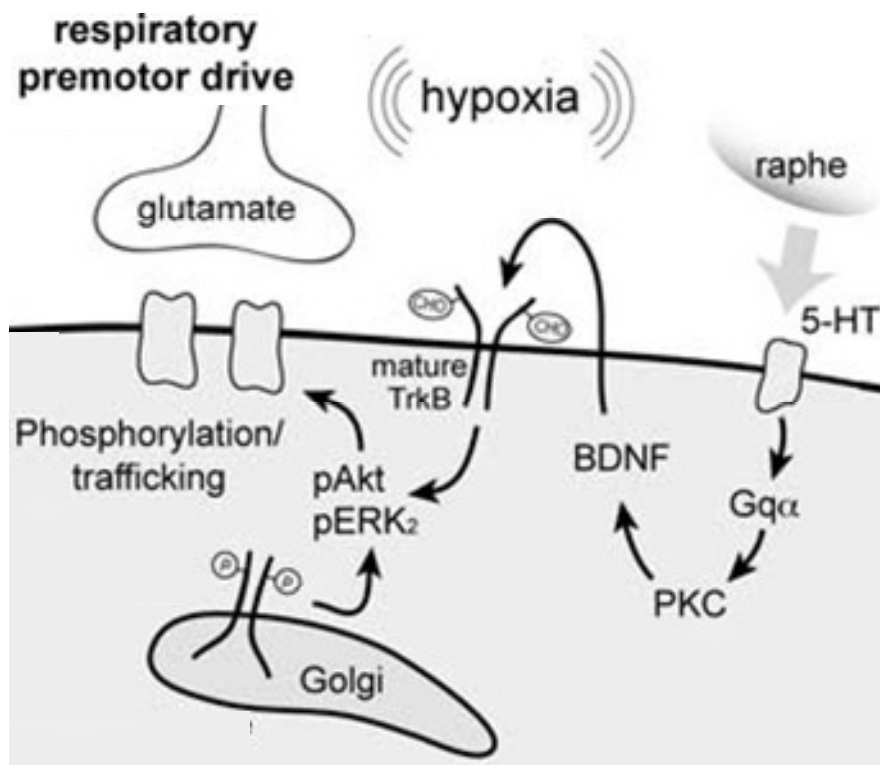


Figure 1.5: Schematic demonstrating the proposed responsible mechanistic pathway for phrenic LTF. 5-HT₂ receptors on respiratory motoneurons are activated by IH-mediated spinal serotonin release from medullary raphe serotonergic neurons. Subsequent spinal protein synthesis (e.g. brain-derived neurotrophic factor - BDNF) activates its endogenous high affinity receptor tyrosine kinase (TrkB) which maintains phrenic LTF. Schematic adapted from Mahamed & Mitchell (2007).

1.5.4 Respiratory LTF in humans

At present we are some way from understanding how best to utilise respiratory LTF as a therapeutic strategy for respiratory conditions such as OSA and if it is beneficial at all. However, because of the promising evidence from animal studies demonstrating respiratory LTF of considerable magnitude, a growing body of

translational human research has also been performed and is attracting increasing interest.

The first published study to specifically investigate respiratory LTF in humans was 16 years after the phenomenon was first reported in animals (McEvoy et al., 1996). Eleven awake male volunteers were exposed to ten 2-minute hypoxic episodes (S_aO_2 80%) that were separated by two-minute intervals of euoxia whilst isocapnia was maintained at baseline levels throughout. Minute ventilation (\dot{V}_E), diaphragmatic activity and genioglossus muscle activity were measured at baseline, during acute IH and for ten minutes during recovery. However, LTF was not demonstrated in any of these respiratory variables suggesting that in contrast to animals acute IH is not a stimulus capable of eliciting respiratory LTF in awake humans. Of course one negative finding does not categorically rule out the existence of respiratory LTF in humans and scientists continued to attempt to evoke respiratory LTF in numerous studies that followed. During the next decade the effect of gender, the duration of hypoxic exposures, the intensity of hypoxic exposures, duration of recovery and previous exposure to IH on the development of respiratory LTF were all examined without any form of respiratory LTF being reported in healthy awake humans (Mateika & Sandhu, 2011). There was only slightly more success in studies of sleeping humans where ventilatory LTF was at least reported in individuals with inspiratory flow limitations (Babcock and Badr, 1998) and LTF of upper airway dilator muscle activity in OSA patients (Aboubakr et

al., 2001). It wasn't until 2006 when respiratory LTF was first reported in healthy humans where the requirement for elevating CO_2 was demonstrated (Harris et al., 2006).

Requirement for Pa_{CO_2} being above the chemoreflex threshold

Based on previous animal studies that highlighted the importance of Pa_{CO_2} levels on the expression of respiratory LTF, it was postulated that previous unsuccessful attempts to induce respiratory LTF in awake humans may be due to Pa_{CO_2} levels being below the central and peripheral chemoreflex thresholds. During wakefulness in humans Pa_{CO_2} levels below the ventilatory threshold have little or no effect on ventilation. In contrast to sleep, this reduction in chemoreflex-initiated ventilatory drive does not lead to the abolishment of ventilation as it can be sustained by arousal and/or behavioural stimuli. Therefore, under normal Pa_{CO_2} conditions during wakefulness the responsible mechanism(s) for mediating respiratory LTF could have been activated during acute IH but the expression of respiratory LTF may have been restrained because ventilation was not controlled by the chemoreflex. By raising the end-tidal partial pressure of CO_2 (PET_{CO_2}) 5 mmHg above normal wakefulness levels and maintaining this throughout acute IH and recovery, ventilatory LTF and LTF of genioglossus muscle activity was expressed in awake humans (Harris et al., 2006). A number of subsequent studies by the same group have since replicated these results and also demonstrated the existence of ventilatory LTF in women and OSA patients (Wadhwa et al., 2008,

Lee et al., 2009, Gerst et al., 2011). Prior to the study by (Harris et al., 2006) respiratory LTF of any form had not been demonstrated in healthy sleeping humans without upper airway flow limitations (e.g. snoring or OSA) despite numerous attempts (Mateika and Sandhu, 2011). Ventilation during sleep is completely controlled by the chemoreflex and thus it might be expected that respiratory LTF would have been expressed following exposure to IH without having to raise PaCO_2 levels in these studies. However, exposure to hypoxia increases ventilation and drives down PaCO_2 levels which may have impaired the expression of respiratory LTF. Indeed, in future studies that gave supplemental CO_2 during hypoxic episodes to prevent the hypoxic-induced hypocapnia reported both ventilatory LTF (Pierchala et al., 2008) and LTF of genioglossus muscle activity (Chowdhuri et al., 2008) in healthy, non flow-limited individuals.

Is respiratory LTF in humans pattern sensitive?

Since the initial study by (Harris et al., 2006) demonstrated the requirement for supplemental CO_2 for the expression of respiratory LTF, many more studies have reported IH-induced respiratory LTF in hypercapnic awake humans (Lee et al., 2009, Wadhwa et al., 2008, Gerst et al., 2011, Diep et al., 2007) and in isocapnic sleeping humans (Pierchala et al., 2008, Chowdhuri et al., 2008). However, it has not been investigated if under these elevated PaCO_2 conditions acute CH can also evoke respiratory LTF. Although animal evidence strongly suggests the

manifestation of respiratory LTF requires an intermittent pattern of hypoxic exposure it cannot be assumed that humans will respond in the same way.

Mechanisms of respiratory LTF

As a result of the invasive nature of the majority of techniques used in animal studies to gain mechanistic insights into the development of respiratory plasticity only limited translational research has been undertaken in humans. Furthermore, the initial demonstration of respiratory LTF in humans occurred relatively recently (Harris et al., 2006) in comparison to that of animals (Millhorn et al., 1980a). As a result the limited number of studies investigating respiratory LTF in humans have primarily focused on confirming its existence in different genders (Wadhwa et al., 2008), in OSA patients (Lee et al., 2009) and examining whether time of day and repeated exposures alters its magnitude (Gerst et al., 2011). (Lee et al., 2009) exposed OSA patients and healthy controls to acute IH in order to examine whether ventilatory LTF was enhanced in OSA patients. Their results demonstrate that respiratory LTF was augmented in OSA patients and also showed that the augmentation was abolished by systemic administration of antioxidants. This data supports evidence from animal studies that have demonstrated prior exposure to chronic IH augments respiratory LTF and that it is mediated by ROS (Del Rio et al., 2010, MacFarlane et al., 2008). However, because antioxidant administration only abolished the augmentation in respiratory LTF in OSA patients but did not affect that occurring in healthy controls the mechanisms responsible for initiating and maintaining respiratory LTF following acute IH in healthy humans remain unknown.

1.5.5 Progressive augmentation of the HVR

Research into progressive augmentation of the HVR has received far less attention than respiratory LTF, presumably because in contrast to respiratory LTF it is widely considered that it may have a detrimental rather than a beneficial effect on breathing stability in OSA patients. The first indication of progressive augmentation of the HVR was reported in inspiratory intercostal nerve and phrenic activity of anesthetized cats during intermittent carotid sinus nerve stimulation (Fregosi and Mitchell, 1994). Progressive augmentation of phrenic and hypoglossal nerve activity of anesthetized rats has since been reported during acute IH (Fuller, 2005). Furthermore, progressive augmentation of \dot{V}_E during successive hypoxic episodes has been reported in awake goats (Turner and Mitchell, 1997) awake ducks (Mitchell et al., 2001b) and anaesthetized rabbits (Sokolowska and Pokorski, 2006). However, there also exist studies that report that progressive augmentation of the HVR is not evident during acute IH in animals (Olson et al., 2001, McGuire et al., 2002).

Requirement for CO₂

As is the case with respiratory LTF following IH the demonstration of progressive augmentation of the HVR in some studies and its absence in others may be due to the level of PaCO₂. Indeed, all animal studies that demonstrated progressive augmentation of the HVR utilised supplemental CO₂ to prevent hypoxia-induced hypocapnia (Mateika and Narwani, 2008). Furthermore, in early human studies

investigating respiratory plasticity P_{ETCO_2} during hypoxic exposures was kept at, or below normal levels, which may explain why progressive augmentation of the HVR as measured by \dot{V}_E and/or genioglossus activity was not demonstrated (McEvoy et al., 1996, Jordan et al., 2002, Mateika et al., 2004, Khodadadeh et al., 2006). When mild hypercapnia was sustained throughout hypoxic exposures progressive augmentation of both \dot{V}_E and genioglossus activity was expressed (Harris et al., 2006) with the former having since been confirmed in other studies (Wadhwa et al., 2008, Lee et al., 2009, Gerst et al., 2011).

The relationship between the HVR and Pa_{CO_2} is important because progressive augmentation of the HVR may be due to alterations in either the ventilatory recruitment threshold or peripheral sensitivity (Duffin, 2007). Therefore, as ventilation in human studies was assessed during successive hypoxic exposures at a single level of hypercapnia (Wadhwa et al., 2008, Lee et al., 2009, Gerst et al., 2011) it is not clear if the progressive augmentation of the HVR was due to an enhancement of chemosensitivity (i.e. sensitivity to Pa_{CO_2}) or whether the ventilatory recruitment threshold (i.e. when ventilation begins to increase in a linear fashion to elevated Pa_{CO_2} having had little effect at lower levels of Pa_{CO_2}) is lowered. To determine whether the recruitment threshold is altered it is necessary to perform a hypoxic chemosensitivity test where Pa_{CO_2} is progressively increased from a significantly hypocapnic level and thus the ventilatory threshold can be determined. Three studies on humans have been undertaken where such tests

were performed before and after exposure to IH (Morelli et al., 2004, Mateika et al., 2004, Ahuja et al., 2007). In all three studies chemosensitivity during hypoxia was increased without a change in the ventilatory threshold.

Is progressive augmentation of the HVR the same phenomenon as respiratory LTF and what is the responsible mechanism(s)?

As discussed previously, it is commonly believed that respiratory LTF may mitigate OSA by facilitating ventilation and upper airway muscle activity, whereas progressive augmentation of the HVR is more likely to promote OSA by promoting hypocapnia and reducing ventilatory drive (Mateika and Narwani 2008). If artificially inducing or enhancing respiratory LTF is to be used as a successful treatment for OSA it will need to occur in the absence of progressive augmentation of the HVR. However, it has been suggested that progressive augmentation of the HVR and respiratory LTF could be the same form of respiratory plasticity just demonstrated under different O₂ tensions but mediated by the same mechanism(s) (Richerson, 2010).

It is well understood in animals that respiratory LTF is mediated by a serotonin dependant spinal cord mechanism as discussed previously (see: *mechanisms of respiratory LTF*). Interestingly, (Fregosi and Mitchell, 1994) reported that although pre-treatment with a serotonergic antagonist (methysergide) abolished the development of respiratory LTF in cats following intermittent carotid sinus nerve stimulation it did not affect the expression of progressive augmentation of phrenic

and intercostal nerve activity. These results would suggest that progressive augmentation of the HVR is independent of respiratory LTF as they require different mechanisms for their development. Because the CB plays a predominate role in numerous time dependant ventilatory responses to hypoxia such as acclimatisation to CH (Powell et al., 1998) it seems logical that the CB would contribute to the progressive augmentation of the HVR following IH. However, an interesting finding from the study by (Fregosi and Mitchell, 1994) is that progressive augmentation of phrenic and intercostal nerve activity manifest during intermittent carotid sinus nerve stimulation independent of the CB being exposed to IH. This would suggest that progressive augmentation of the HVR can be evoked independently of a change in peripheral chemoreflex sensitivity. These findings are supported by (Peng et al., 2003) who demonstrated that both *in* and *ex vivo* CB afferent discharge did not change from the first to the last hypoxic episode during a pattern of IH previously shown to evoke progressive augmentation of the HVR. No animal study has since been undertaken to further investigate what is the responsible mechanism(s) for progressive augmentation of the HVR. All we can conclude from the available animal literature is that progressive augmentation of the HVR is mediated independent of a change in CB chemoreflex responsiveness or a serotonin dependant mechanism in the spinal cord.

Even less has been learnt from human studies regarding the mechanisms for progressive augmentation of the HVR as invasive techniques performed in animal studies cannot be replicated (e.g. carotid sinus nerve stimulation and application of

serotonin inhibitors). The most informative method that can be used is to perform two different CO₂ sensitivity tests. One test is performed under hypoxic conditions to determine the combined contribution of the central and peripheral chemoreflex to the HVR and the other under hyperoxic conditions to determine the independent contribution of the central chemoreflex. These tests have been performed in three separate studies on humans before and after exposure to IH (Morelli et al., 2004, Mateika et al., 2004, Ahuja et al., 2007). It was shown in all three studies that combined central and peripheral chemoreflex sensitivity was significantly increased following IH. A smaller independent increase in central chemoreflex sensitivity was shown in all studies but only in one of these studies was it statistically significant (Morelli et al., 2004). In Mateika's et al. (2004) study the increase in central chemosensitivity was subtracted from that of the larger increase in the combined central and peripheral chemosensitivity. Because there remained a significant increase in sensitivity after the subtraction of the central component, the authors claim that exposure to IH has increased peripheral chemosensitivity. However, this assumption relies on the resulting ventilatory response of the peripheral and central chemoreflexes being simply additive, which is not the case. Indeed, (Blain et al., 2010) recently showed that in dogs there is a substantial hyperadditive interaction between the peripheral and central chemoreflexes. Therefore, it cannot be concluded that IH did not enhance this hyperadditive interaction between central and peripheral chemoreflexes without actually altering peripheral chemosensitivity. Furthermore, as a result of this substantial hyperadditive interaction it cannot be confirmed that the modest increase in central

chemosensitivity shown in all studies does not account for the majority of the increase in chemosensitivity when the peripheral chemoreflex is also active.

1.5.6 Summary of respiratory plasticity

The potential for harnessing or artificially evoking IH-induced respiratory LTF as a treatment for conditions associated with respiratory insufficiency has certainly attracted much interest from scientists over the last three decades. As a result of this extensive research it would appear that we have greatly expanded our understanding of the responsible mechanisms of respiratory plasticity. Indeed, scientists can now induce respiratory LTF in animals by administering specific proteins into the spinal cord (Hoffman and Mitchell, 2011) in the absence of exposure to IH. Therefore, it would seem likely that animal research will now focus on being able to administer these specific proteins intravenously and if successful the next step will be to test their effectiveness in humans. Sceptics of inducing respiratory LTF as a treatment for OSA will argue that there is no evidence to suggest that respiratory plasticity will be beneficial to breathing stability. Although this is a valid argument, it may be that the magnitude of respiratory plasticity that is developed during OSA may be too small to have any noticeable effect but greater magnitudes of artificially induced respiratory LTF may be beneficial. However, there also exist a number of other concerns regarding the potential benefit of inducing respiratory LTF via pharmaceutical aids that have been developed based on our understanding of respiratory LTF in animals. Overwhelming evidence has indicated that a central mechanism mediates respiratory plasticity in animals and

studies have identified the exact proteins (e.g. BDNF) in the spinal cord that are responsible for its manifestation. However, in contrast to the huge body of animal research regarding IH-induced respiratory plasticity there exists only very limited translational human research. Indeed, at present there is not a single study that has been performed to confirm that respiratory LTF is mediated by a central mechanism in humans. If in fact a change in CB activity maintains respiratory LTF in humans, then strategies to increase concentrations of proteins such as, BDNF in the spinal cord might not have the same effect on respiration as it does in animals. Furthermore, there is convincing evidence in animals that respiratory LTF is pattern sensitive and that only IH or intermittent serotonin receptor activation evokes respiratory LTF. However, since it was demonstrated in humans that elevating PaCO_2 was a requirement for the manifestation of respiratory LTF following acute IH it has not been examined whether acute CH also causes respiratory LTF under these conditions.

1.6 Aims of this thesis and proposed studies

This thesis consists of one methodological chapter and five experimental chapters. The first aim of the thesis was to develop a new airway occlusion model of OSA that could be used to better investigate the effects of OSA-related IH. The development of this model is described in detail in chapter 2. The first experimental study (chapter 3) examined the success of using Doppler ultrasound to investigate

the effects of IH as it occurs in OSA on pulmonary haemodynamics in humans using the new model of airway occlusions. Also using the new model of airway occlusions in the second study (chapter 4), we aimed to investigate the manifestation of respiratory plasticity following hypoxic airway occlusions and to investigate whether exposure sensitises HPV in humans. By reducing the P_{IO_2} in the third study (chapter 5) we aimed to investigate whether acute CH in addition to acute IH can induce respiratory LTF in humans and what were the responsible mechanisms (e.g. changes in peripheral or central chemosensitivity). In the fourth study (chapter 6) we examined the effects of simultaneous hyperglycaemia on oxidative stress during IH as induced using an airway occlusion model of OSA in rats. Finally, in the fifth study (chapter 7) we investigated whether simultaneous exposure to combined postprandial hyperglycaemia and hyperlipidaemia increased oxidative stress during exposure to intermittent reductions in P_{IO_2} in humans.

1.6.1 Primary questions investigated in this thesis

- 1) Can a new human airway occlusion model of OSA be successfully implemented in experimental research investigating the cardiovascular and respiratory effects of IH as occurs in OSA?
- 2) What is the contribution of hypoxia to the elevation in PAP during apnoeas and does IH sensitise hypoxic pulmonary vasoconstriction in humans?
- 3) Does exposure to hypoxic airway occlusions elicit respiratory plasticity in humans?

- 4) During sustained mild hypercapnia does ventilatory LTF manifest in awake humans following acute exposure to CH?
- 5) Is ventilatory LTF following acute IH and/or acute CH actively maintained by the CB?
- 6) Does simultaneous exposure to hyperglycaemia augment oxidative stress during hypoxic airway occlusions in rats?
- 7) Is oxidative stress during acute exposure to IH augmented by simultaneous exposure to combined postprandial hyperglycaemia and hyperlipidaemia in humans?

2 CHAPTER 2 – GENERAL METHODS

2.1 Gas control

Experiments were undertaken in this thesis to examine the effects of OSA-related IH on various physiological systems. The study of the effects of a gas in the body depends on its presence in arterial blood and in order to induce arterial hypoxia it is necessary to manipulate (lower) the $P_{I_{O_2}}$. Therefore, in experiments studying OSA and the effects of the associated IH, scientists have used protocols during which participants breathe alternating gas mixtures of low and normal $P_{I_{O_2}}$. However, humans vary greatly in their ventilatory response to a set $P_{I_{O_2}}$ and thus the resulting $P_{a_{O_2}}$ will also vary substantially. Furthermore, increased ventilation during hypoxia is not accompanied by a parallel elevation in metabolic rate and thus hypocapnia manifests. Therefore, implicating the physiological effects to hypoxia is complicated by hypocapnia, which may cooperate or compete with it. Although increasing the P_{CO_2} of the inspired gas mixture can be used in an attempt to abolish hypocapnia, the amount of inspired P_{CO_2} that is needed for this purpose also depends on the individual ventilatory response to hypoxia. For this reason, eucapnic conditions that are maintained accurately are hard to achieve with set inspired gas mixtures. In order to overcome the limitations of using a set inspired stimuli, Swanson and Bellville 1975 developed a computer-controlled prediction-

correction scheme, further developed by (Robbins et al., 1982) called dynamic end-tidal forcing (DEF).

2.1.1 Dynamic end-tidal forcing

The DEF system allows PA_{O_2} and PA_{CO_2} to be tightly maintained at desired values despite the variation in respiratory responses to these respiratory stimuli between individuals. A fundamental principle of the DEF system is the assumption that an equilibration between alveolar PO_2 and PCO_2 and that of arterial blood returning to the heart is achieved. Measuring PET_{O_2} and PET_{CO_2} allows an indirect measurement of arterial PO_2 and PCO_2 . In order to evoke the desired PET_{O_2} and PET_{CO_2} values in an individual, a formula based on models of the circulation and the respiratory system is used to predict the required inspired gas composition. During experiments the actual values of PET_{O_2} and PET_{CO_2} are measured for each breath and they are compared to the desired values. A correction scheme is then utilised to alter the initial prediction for the subsequent breath based on the difference between the actual and desired end-tidal values. In all experiments the prediction and correction elements of the DEF system (as described below) were applied using PC-based software (BreathM, Oxford, UK).

Prediction: Calculations to predict the inspired gas compositions that are required to generate the desired end-tidal profiles are performed in advance of each experiment. These calculations are initially derived from the mass balance

equations of O₂ and CO₂ in the lungs providing an equation whereby the inspired partial pressure (P_I) of the gas in question can then be derived as shown below (equation 1) (Robbins et al., 1982),

$$P_I = \frac{V_A P_A + V_L \cdot \frac{dP_A}{dt} + \lambda Q (C_a - C_{\bar{v}})}{V_A} \quad \text{Equation 1}$$

where \dot{V}_A is alveolar ventilation, P_A is the partial pressure of the gas in question in the alveoli, V_L is the lung volume, λ is a coefficient dependent on units, \dot{Q} is pulmonary blood flow, C_a is the arterial concentration of the gas in the blood, and $C_{\bar{v}}$ is the concentration of the gas in question in mixed venous blood. This equation is then solved using a two-component model of the respiratory control system that includes the peripheral and central chemoreflex and a multi-component model of the circulatory system.

Correction: An *integral-proportional* feedback system is used to make corrections to the predictive inspired gas compositions based on the difference between desired end-tidal values and the actual values measured for each breath as shown below (equation 2),

$$P_{I(n)} = P_{I_C(n)} + g_p \cdot (P_{ET_d(n-1)} - P_{ET_m(n-1)}) + g_i \cdot \left(\sum_{j=1}^{n-1} (P_{ET_d(j)} - P_{ET_m(j)}) \right) \quad \text{Equation 2}$$

where P_{I_C} is the predicted inspired partial pressure, P_{ET_d} is the desired end-tidal partial pressure, P_{ET_m} is the measured end-tidal partial pressure, g_p is the

proportional feedback gain, and g_i is the integral feedback gain. *Proportional* feedback is calculated from the difference between actual and desired end-tidal values from the last breath and it is used to reduce breath-to-breath variation whereas *integral* feedback is calculated from the sum of the differences from all previous breaths. Both gains can be adjusted manually during experiments to generate greater accuracy.

2.2 Development of a novel airway occlusion model of OSA

2.2.1 General

Research investigating whether IH might mediate the development and progression of cardiovascular disease in OSA patients has predominantly been performed in animals. These studies have most commonly elicited IH independent from other stimuli occurring in OSA by simply intermittently reducing the PI_{O_2} . Although these studies have supplied valuable data highlighting the potential risk to health from exposure to IH, this approach is limited in its success in mimicking IH caused by OSA (see: *General introduction*). Therefore, recent studies have begun utilising a variety of airway occlusion models to investigate the pathology of OSA-related IH (Simpson et al., 2008, Othman et al., 2010, Schoorlemmer et al., 2011). However, until now only limited research has been completed in humans with these studies evoking IH by reducing the PI_{O_2} or using repetitive breath-holds to rather weakly mimic OSA. Further human research is required to better understand

the pathological consequences of OSA-related IH and an accurate laboratory model of OSA may enhance future research. Therefore, we attempted to design a model of OSA whereby awake humans could be exposed to as many of the different components of OSA as possible with or without simultaneous hypoxia. We hoped that this model could then be used in future studies throughout this thesis to investigate the effects of OSA-related IH on various physiological variables such as, ventilation, oxidative stress, pulmonary and systemic haemodynamics etc.

2.2.2 Existing models of OSA and identification of potential improvements

We are not the first to attempt to develop a model of OSA in awake humans as scientists realised early on that exposure to reduced P_{IO_2} may not be the best approach to investigate the effects of IH that occur in OSA. Indeed, a number of studies have previously been performed using breath-holds in awake humans to better mimic an apnoeic event (Cutler et al., 2004a, Cutler et al., 2004b, Leuenberger et al., 2007, Leuenberger et al., 2005). Cutler and colleagues were the first to study the effects of repetitive breath-holds and had participants perform them for 20 seconds on end-expiration, every minute for 20 minutes (Cutler et al., 2004b, Cutler et al., 2004a). Each breath-hold was preceded by two breaths of 100% N_2 to augment the drop in S_aO_2 during the breath-hold and induce post-apnoeic S_aO_2 nadirs in the range of 80-85%. The only alteration from this design used in subsequent studies by Leuenberger and colleagues (2005 & 2007) was that the two breaths of 100% N_2 were replaced with 20 seconds breathing 10% O_2 .

In all four of these breath-hold studies participants were requested to briefly exhale following each 20-second apnoea to measure PET_{CO_2} . This demonstrates a significant elevation from pre breath-hold values and thus participants are exposed to intermittent hypercapnia in addition to IH as occurs during obstructive apnoeas in OSA. Furthermore, these hypoxic breath-holds induced transient elevations in sympathetic activity and systemic blood pressure and evoked hyperventilation and tachycardia immediately post breath-hold that are all known to occur during an apnoeic cycle in OSA.

These hypoxic breath-hold models of OSA are certainly an improvement from simply exposing individuals to reductions in P_{IO_2} . However, during an obstructive apnoea patients continue to produce inspiratory attempts against the occluded airway that become more frequent and progressively greater in force as the apnoea continues. Each occluded inspiratory effort generates negative intrathoracic pressure that has been reported to fall as low as -65 mmHg (Malone et al., 1991). These brief but severe negative intrathoracic pressures have a number of significant haemodynamic effects on the heart. Firstly, venous return is augmented as a result of the large pressure gradient generated between the extra and intrathoracic space (Moreno et al., 1967). Secondly, studies on OSA patients have demonstrated reduced SV during occluded inspiratory efforts (Tolle et al., 1983). Furthermore, a recent study in humans using echocardiography demonstrated that the decrease in SV during negative intrathoracic pressure is a

result of decreased ejection fraction and is subsequently associated with elevations in left ventricular afterload (Orban et al., 2008). The pathological consequences of these haemodynamic disturbances are not fully understood although it is known that the resulting repetitive congestion of blood in the pulmonary system transiently increases PAP (Marrone et al., 1994, Schafer et al., 1998). Furthermore, the same study by (Orban et al., 2008) demonstrated that left atrial volume fell during negative intrathoracic pressures but rapidly increased above baseline during resumption of normal intrathoracic pressure (Orban et al., 2008). The physiological effects of these frequent elevations in left atrial volume are currently unknown but the authors suggest that the atrial wall stretch could be significant enough to account for the elevated levels of atrial natriuretic peptide that OSA patients experience during the night.

Based on the aforementioned evidence that negative intrathoracic pressures can cause significant swings in chamber volumes and affect PAP it is plausible that any detrimental effect of IH on the cardiovascular system may be augmented when simultaneous to these negative intrathoracic pressures. We felt that occluded inspiratory efforts should be incorporated into our model of OSA.

Another potential improvement to existing models of OSA would be the inclusion of a more accurate method to control the level of hypoxia occurring during airway occlusions. As previously discussed, in existing models of OSA participants are

exposed to a brief period of reduced P_{iO_2} immediately prior to performing a breath-hold (Cutler et al., 2004a, Cutler et al., 2004b, Leuenberger et al., 2007, Leuenberger et al., 2005). This is required because a brief 20-second breath-hold in awake humans who were previously breathing room air evokes only a very small decrease in S_aO_2 (Leuenberger et al., 2005). In contrast, S_aO_2 in OSA patients frequently falls into the low 80s (%) and in severe cases can fall into the low 50s (%) (Sforza et al., 1998). This substantial difference in S_aO_2 nadirs between wakefulness and sleep may be due to a combination of the following: lower resting P_{aO_2} levels during sleep vs. wakefulness; reduced airflow pre apnoea due to partial upper airway collapse (hypopnoea); longer duration of apnoeas than the 20 seconds that can be performed in awake humans; and incomplete re-oxygenation between apnoeas.

When developing our airway occlusion model of OSA we noticed that the drop in P_{ETO_2} (that in healthy individuals represents P_{aO_2}) following two breaths of 100% N_2 varied greatly between and within individuals and it was influenced by the individual's tidal volume (TV). Figure 2.1 shows original data from two participants that demonstrate very different P_{ETO_2} values following two breaths of 100% N_2 . Furthermore, the P_{aO_2} generated from breathing a set inspiratory gas composition for longer periods (e.g. 20 seconds of 10% O_2) is dependent not only on the gas composition but also on the ventilatory response that ensues which can vary greatly between individuals. This variation in P_{aO_2} prior to airway occlusions could

have important consequences on the severity of post airway occlusion nadirs in S_aO_2 . The oxygen-haemoglobin dissociation curve demonstrates a sigmoidal shape and thus a small change in Pa_{O_2} (± 10 mmHg) around a pre airway occlusion value of Pa_{O_2} of 80 mmHg has little effect on S_aO_2 (~3%). However, the same change (± 10 mmHg) around a lower post airway occlusion Pa_{O_2} of 50 mmHg has a much greater effect on S_aO_2 (~12%). Therefore, to ensure post airway occlusion nadirs in S_aO_2 were similar between individuals and similar between repetitive airway occlusions in the same individual we felt that PET_{O_2} should be clamped at an individually tailored level prior to each airway occlusion.

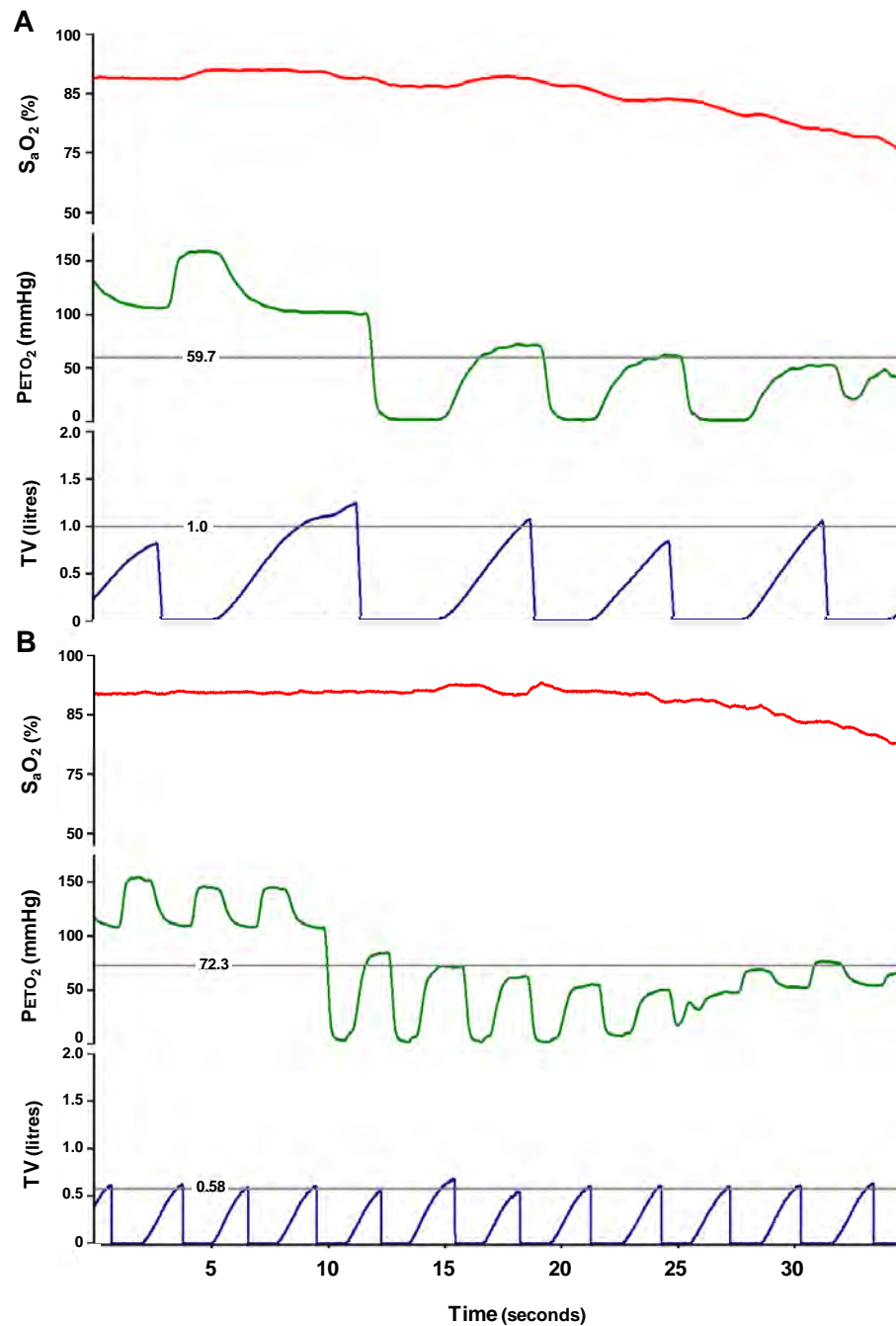


Figure 2.1: Original data showing S_aO_2 , $PETO_2$ and tidal volume (TV) during exposures to 100% inspired N_2 in two participants. Note how $PETO_2$ falls to ~60 mmHg after two breaths of 100% N_2 in the participant with a large TV (**A**) and just ~72 mmHg in the participant with a small TV (**B**).

In a previous study by (Leuenberger et al., 2005) to determine whether IH was responsible for any of the physiological effects caused by exposure to hypoxic breath-holds, participants were also exposed to breath-holds that were preceded by room air breathing rather than reductions in $P_{I_{O_2}}$. However, as a small but significant drop in S_{aO_2} still developed during these breath-holds the success of using these breath-holds following room air breathing as a control trial is restricted. Although acute exposures to this mild IH may not be sufficient to evoke any physiological effects independently, it should be considered that it may still greatly enhance the effects from other simultaneous stressors such as hypercapnia and haemodynamic stress during negative intrathoracic pressures. Therefore, we felt that airway occlusions used in any control trials should be preceded by mild hyperoxia to completely abolish any drop in S_{aO_2} . As with inducing mild hypoxia prior to airway occlusion we felt that the elevation in $P_{ET_{O_2}}$ during hyperoxia should be clamped at an individually tailored level to ensure post airway values of $P_{ET_{O_2}}$ are consistent between individuals.

A final point to consider in the design of a model to mimic OSA is that of P_{aCO_2} . During sleep P_{aCO_2} is higher than during wakefulness and in patients with OSA P_{aCO_2} may also rise further during reductions in airflow before an apnoeic event due to a partial collapse of the upper airway (hypopnoea). Therefore, the post-apnoeic P_{aCO_2} is greater during sleep than following a breath-hold of the same duration during wakefulness. The existing hypoxic breath-hold studies not only

ignored the issue of the level of hypercapnia following a breath-hold but may also have caused it to be even lower by using poikilocapnic hypoxia before each airway occlusion (Cutler et al., 2004a, Cutler et al., 2004b, Leuenberger et al., 2007, Leuenberger et al., 2005). Therefore, we considered introducing a small elevation in PaCO_2 prior to airway occlusions would better mimic the magnitude of intermittent hypercapnia experienced in OSA.

For artificially induced airway occlusions to most accurately mimic the cycle of apnoeas occurring in OSA these airway occlusions should be elicited during sleep. This would allow both the post apnoeic arousals and the subsequent transient elevations in sympathetic activity to be replicated. However, although occluding the airway during sleep could theoretically be achieved by wearing a facemask and briefly causing an obstruction to the external airway (tubing), we believed it would be unsafe and would cause complete arousal from sleep rather than the partial arousal that occurs in OSA. Furthermore, the ability to perform experimental measurements such as those of PAP using Doppler ultrasound would not be possible with sleeping participants. Therefore, we were restricted to developing an airway occlusion model of OSA in awake humans.

Summary of suggested improvements to existing models of OSA

- 1) Addition of inspiratory efforts against an occluded airway that mimic both the increasing frequency and increasing negative intrathoracic pressures that occur during an obstructive apnoea.
- 2) Tailor the degree of hypoxia prior to airway occlusions in each individual to allow tight control of post airway occlusion nadirs in S_aO_2 .
- 3) Elicit mild hyperoxia prior to airway occlusions and tailor the degree of hyperoxia to each individual in order to completely inhibit the development of hypoxia and thus provide a successful control for hypoxic airway occlusions.
- 4) Introduce a small elevation in $PETCO_2$ prior to airway occlusions in order to better mimic the degree of intermittent hypercapnia that occurs during apnoeas in OSA patients.

2.2.3 Model design

Participant position: Although the airway occlusions could easily be performed whilst sitting, having participants lie down would be more comfortable for longer periods and it would better reflect the body position during sleep. Furthermore we chose to have participants lie on their back as obstruction of upper airway is more common when lying in this position due to gravity pulling the tongue to the back of

throat (except for during ultrasound measurements when it was necessary for participants to turn on their side).

Duration of airway occlusions: Existing human models of OSA have all used breath-holds lasting 20 seconds. We hoped to be able to extend the duration of the breath-holds in order to cause the required degree of hypoxia without having to reduce inspired O₂ prior to airway occlusions. However, although most participants that were involved in the development of the model could perform 30-second breath-holds at end expiration, not all of them could do this without considerable discomfort. Therefore, to ensure we did not introduce a participant selection bias into studies using this model we chose to restrict airway occlusions to 20 seconds.

Frequency of airway occlusions: In order to produce a sufficiently strong stimulus to elicit measurable physiological changes following a relatively short duration of airway occlusions (e.g. several hours per day) we deemed it necessary to mimic the frequency experienced by patients with severe OSA (apnoea hypopnoea index of ≥ 30). Therefore, we chose to induce airway occlusions every 90 seconds which equated to an apnoea hypopnoea index of 40. This selected frequency of airway occlusions still allowed a suitable duration of unrestricted room air breathing between airway occlusions to ensure adequate re-oxygenation and thus induced the intermittent pattern of hypoxia experienced by most OSA patients.

Furthermore, it provided a suitable duration to allow good control of desired PET_{O_2} and PET_{CO_2} prior to the next airway occlusion.

Occluding the airway: In OSA the upper airway collapses during the initial phase of inspiration when negative upper airway pressure is generated. However, it was not feasible to occlude the airway during inspiration in awake humans due to discomfort and thus we decided to occlude the airway at the end of expiration. To this end an inflatable balloon valve (Hands Rudolph, 9340 series) consisting of a small length of translucent tubing with a small silicone rubber balloon in the centre was attached to the breathing circuit near the mouthpiece (see figure 2.2 for a photo of the apparatus). Air flow was continuously measured by a pneumotach positioned beyond the balloon valve and recorded by computer software (Spike2 version 5.21). To induce an airway occlusion at a given time the computer would search for zero flow at the end of the expiration and a signal would be sent to open a solenoid valve. Compressed air would then rapidly inflate the balloon valve which formed a tight seal inside the tubing. The balloon valve would remain inflated for 20 seconds at which point a signal would automatically be sent to close the solenoid valve and the balloon valve would deflate rapidly.

Frequency of occluded inspiratory efforts during an airway occlusion: We were unable to find any published literature concerning the average frequency of occluded inspiratory efforts during apnoeas in OSA patients, only that the

frequency of these efforts increases as the apnoea continues. In theory, it would be possible to measure resting breathing frequency in each participant and base the initial frequency of inspiratory efforts on this. However, it is well known that breathing frequency varies greatly between individuals and thus making comparisons between the effects of airway occlusions between individuals would be complicated by having a different number of occluded inspiratory efforts. Therefore, in the absence of any strong support for a specific number of inspiratory efforts against the occlusion and the increase in frequency of these efforts during the 20-second airway occlusions, we included five inspiratory efforts during the 20-second airway occlusion which progressively increased in frequency as shown in Table 2.1.

Negative intrathoracic pressures: The gold standard for estimating intrathoracic pressures in OSA patients is to use an oesophageal balloon-tipped pressure sensor. However, changes in pressure recorded at the mouth during occluded inspiratory efforts have previously been shown to closely correlate to changes in oesophageal pressure (Baydur et al., 1982). Therefore, a pressure gauge was positioned between the mouthpiece and the balloon valve as shown in figure 2.2. This enabled negative intrathoracic pressures during the occluded inspiratory efforts to be measured and recorded on a computer. During an obstructive apnoea inspiratory efforts gradually increase in force and thus so do the negative intrathoracic pressures that are generated. The greatest nadir in intrathoracic pressure generated during the end of apnoeas varies greatly between OSA

patients as studies have reported values to peak anywhere between -15 mmHg and -65 mmHg (Malone et al., 1991). During the development of this model we asked participants to attempt a range of different negative pressures and concluded that producing pressures much beyond -40 mmHg was uncomfortable. We therefore limited the peak desired intrathoracic pressure to -35 mmHg as shown in table 2.1.

LED signalling panel: To ensure airway occlusions with inspiratory efforts could be successfully and safely performed over a long durations it was imperative that participants were clearly informed when the airway would be occluded and given assistance in producing the desired negative airway pressures during each occluded inspiratory effort. To achieve this, a small panel with two lines of five red and five green LEDs was attached to the breathing circuit as show in figure 2.2. A full description of how this LED panel was used to alert participants to the airway being occluded and help produce the desired negative intrathoracic pressures is described in the next section.

Inspiratory efforts during airway occlusion: Three seconds prior to the desired time for the airway to be occluded (i.e. every 90 seconds) computer software (Spike2 version 5.21) searched for the next inspiration (positive flow from the pneumotach). At that point a signal was sent to the LED panel causing all ten LEDs to flash to alert the participant that at the end of the next expiration the airway will be occluded. Over the course of the airway occlusion red LED's would light up to

indicate when occluded inspiratory efforts should be performed. The number of red LEDs lit at any time indicated the negative intrathoracic pressure that the participant had to generate. One LED indicated a negative pressure of -15 mmHg, two LEDs -20 mmHg, three LEDs -25 mmHg, four LEDs -30 mmHg and five LEDs -35 mmHg. The timings of these red LEDs lighting up are summarised in Table 2.1. Individuals were trained to inspire against the occluded airway at a normal inspiratory speed. The green LEDs were used to aid participants in accurately generating the correct negative intrathoracic pressures and indicating when they should relax after each inspiratory attempt. The negative intrathoracic pressure required to cause each green LED to light up was matched to that of the red LED directly above. Thus, as soon as the number of lit green LEDs matched the number of red LEDs already lit, participants knew they had generated the desired level of negative intrathoracic pressure and could relax.

After 20 seconds of the airway being occluded all of the red and green LEDs would flash to indicate the balloon valve had deflated and that the participant should briefly exhale to allow end-tidal gases to be measured. Participants then breathed freely until the next airway occlusion occurred. Figure 2.3 shows original data from one participant producing these negative intrathoracic pressures. It clearly shows how the magnitude and frequency of the negative intrathoracic pressures increase during the 20 second airway occlusions.

	Airway occluded	1 red LED	2 red LED	3 red LED	4 red LED	5 red LED	Airway open
Time (sec)	0	2	7	11	14	17	20
Interval (sec)		2	5	4	3	3	3
Pressure (mmHg)		-15	-20	-25	-30	-35	

Table **2.1**: Timings of when occluded inspiratory efforts were to be performed during each airway occlusion and the desired negative intrathoracic pressures.

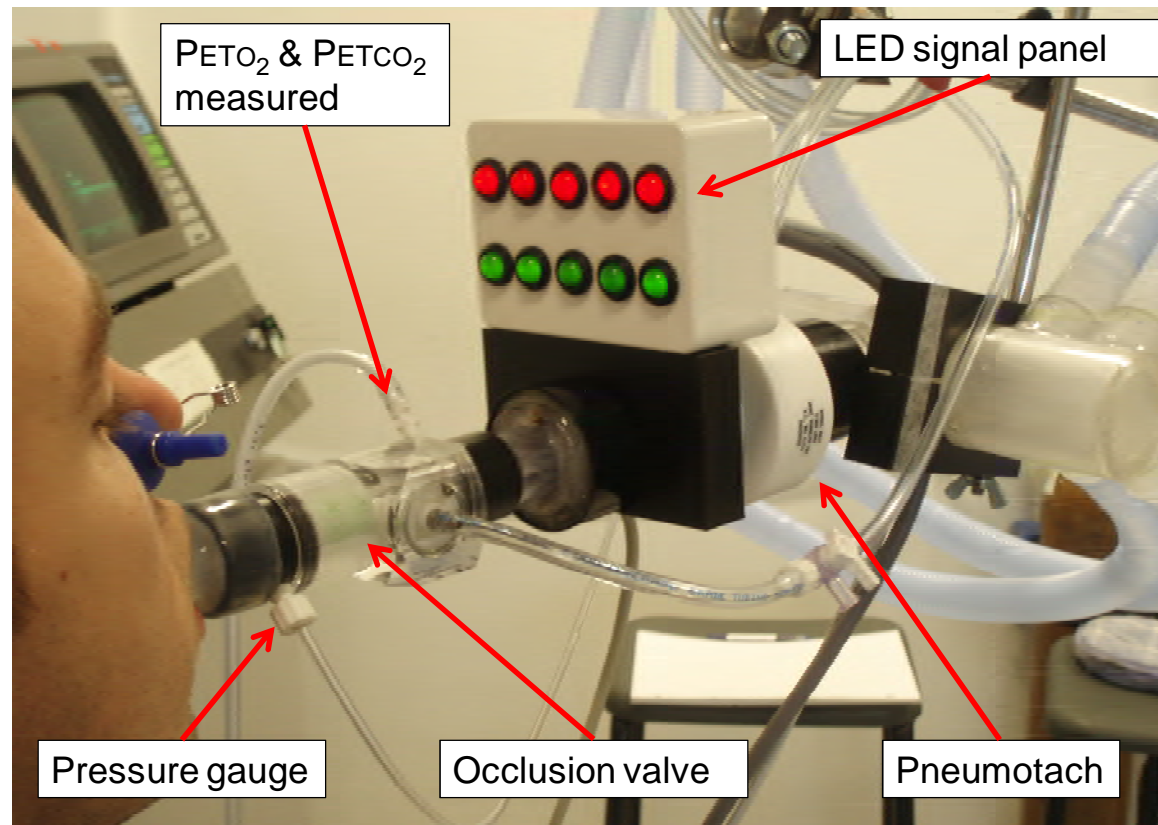


Figure 2.2: A photograph of the experimental setup used to induce airway occlusions.

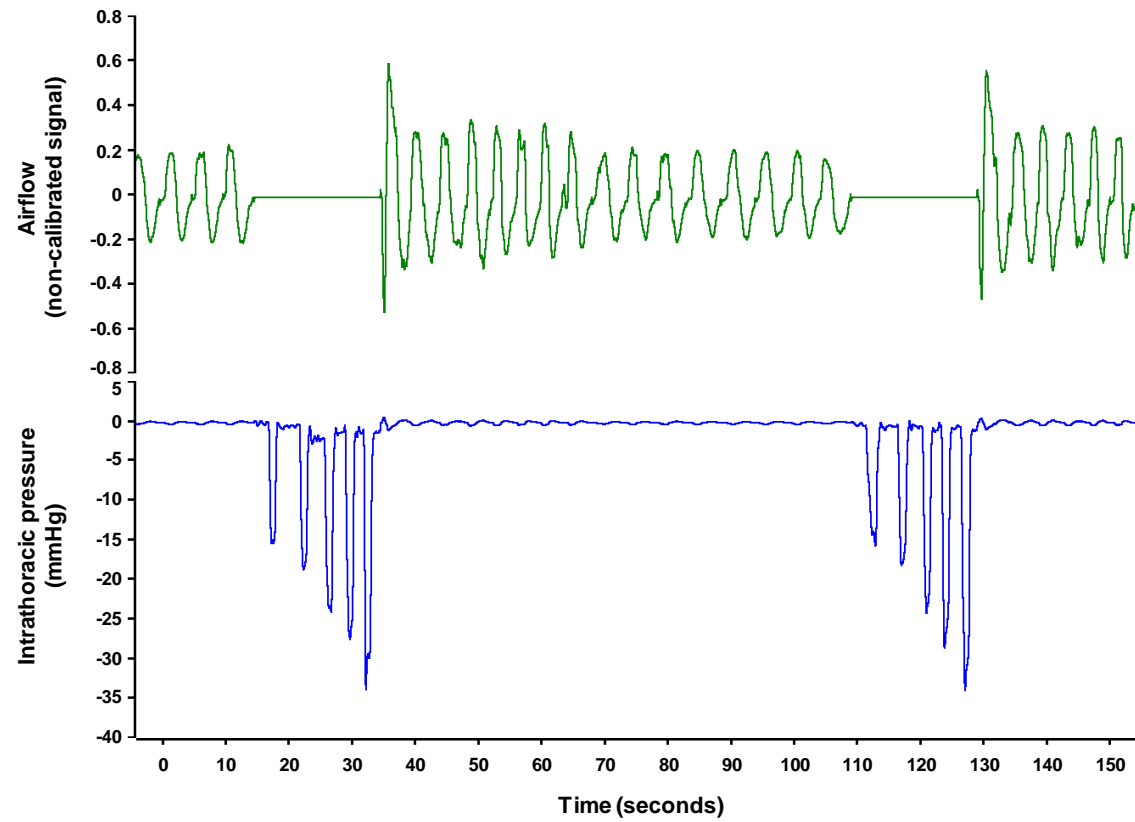


Figure 2.3: Original record for one participant showing air flow and intrathoracic pressure during two successive airway occlusions. Note the absence of airflow during each twenty second airway occlusion and how the negative intrathoracic pressures gradually increase in frequency and magnitude during the time course of the 20-second airway occlusions.

Gas control

Airway occlusions with hypoxia: To enhance hypoxia during airway occlusions we used the DEF system to lower the $P_{ET_{O_2}}$ prior to the occlusion. We found a pre occlusion $P_{ET_{O_2}}$ value of ~70 mmHg would result in a post-occlusion $P_{ET_{O_2}}$ of ~50 mmHg in most individuals. Although $P_{ET_{O_2}}$ could be lowered to 70 mmHg over durations as brief as 10 seconds, there was an insufficient number of breaths to allow the correction scheme of the DEF system to gain tight control of the $P_{ET_{O_2}}$ at 70 mmHg. The most successful method to achieve the desired control was to gradually lower $P_{ET_{O_2}}$ to 70 mmHg over 20 seconds. Small adjustments to the pre-occlusion $P_{ET_{O_2}}$ for each individual allowed a post-airway occlusion $P_{ET_{O_2}}$ of 50 mmHg to be kept consistent between individuals. Figure 2.4a is an original recording of a sequence of hypoxic airway occlusions in one participant. It clearly shows how $P_{ET_{O_2}}$ is clamped at ~70 mmHg prior to airway occlusions resulting in a subsequent post-occlusion nadir in $P_{ET_{O_2}}$ of 50 mmHg.

Airway occlusions without hypoxia: We found that gradually increasing $P_{ET_{O_2}}$ over 15 seconds to a pre-occlusion $P_{ET_{O_2}}$ value of around 145 mmHg ensured that $P_{ET_{O_2}}$ following airway occlusions remained in a euoxic range (~100 mmHg). Figure 2.4b is an original recording of a sequence of airway occlusions without hypoxia. It clearly shows how $P_{ET_{O_2}}$ is elevated to ~145 mmHg prior to airway occlusions and that this results in a post-occlusion $P_{ET_{O_2}}$ of ~100 mmHg that prevented any decline in S_aO_2 .

Enhancing intermittent hypercapnia: In order to increase the magnitude of hypercapnia during airway occlusions to better mimic the magnitude experienced in OSA, the DEF was used to raise PET_{CO_2} ~1 mmHg above a predetermined normal level. This ensured the magnitude of hypercapnia was similar between repetitive airway occlusions in the same individual and thus the acute effects of airway occlusions in the same individual could be compared over time. This small elevation in PET_{CO_2} prior to hypoxic and hyperoxic airway occlusions is demonstrated in the original recordings in figure 2.4.

2.2.4 Model evaluation and implications for future research

In general we were encouraged by how we were able to successfully develop a new airway occlusion model that we feel is superior to existing models of OSA. However, despite the relative ease of performing individual airway occlusions, participants found repeatedly performing them over long periods (of more than one hour) to be both tiring and tedious.

Benefits of the new model: With only minimal coaching and use of the LED display system participants were able to successfully perform occluded inspiratory efforts and produce the desired negative intrathoracic pressures. Furthermore, the DEF system provided an effective tool to enhance or inhibit hypoxia during airway occlusions whilst simultaneously increasing the degree of hypercapnia to a level

that better reflects that of OSA. Finally, we feel the model is effective in replicating the cardiovascular and respiratory responses that occur during repetitive obstructive apnoeas in OSA. Indeed, the original recordings of various physiological variables during a sequence of airway occlusions with and without hypoxia in figure 2.4 clearly demonstrate similar cardiovascular and respiratory responses to those previously shown in the polysomnogram of an OSA patient in figure 1.1. Firstly, there are substantial elevations in ventilation immediately following airway occlusions. Secondly, systemic blood pressure gradually rises during each airway occlusion and peaks upon resumption of breathing. Finally, a significant degree of tachycardia occurs during post airway occlusion hyperventilation.

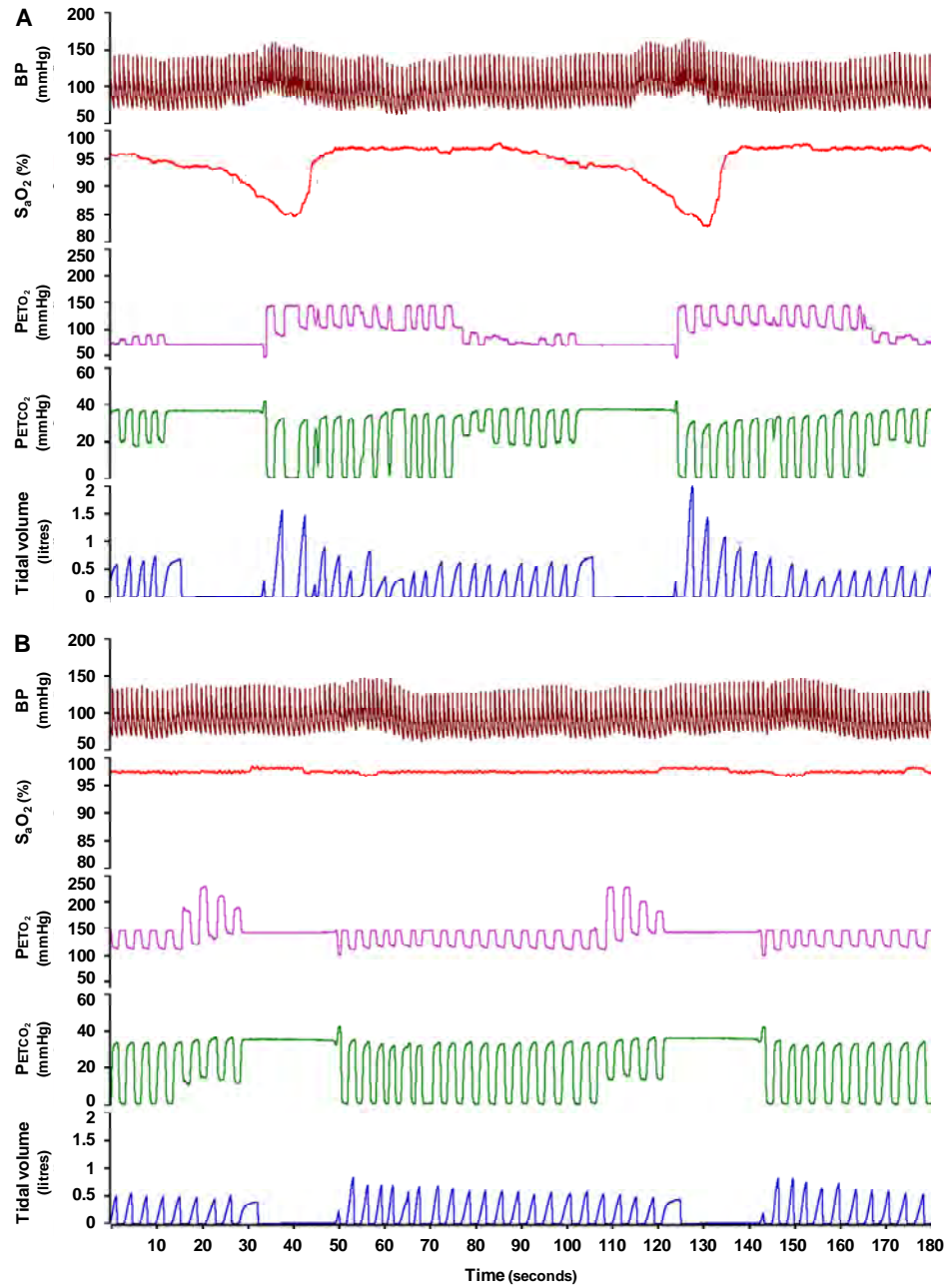


Figure 2.4: Original recordings from one participant showing breath-by-breath $PETO_2$, $PETCO_2$, TV, S_aO_2 and systemic BP during a sequence of airway occlusions with (A) and without hypoxia (B).

Restraints of the new model: Although we opted to use relatively short airway occlusions of 20 seconds which could be performed by all participants, many of them reported feeling physically tired following long exposures to the airway occlusions. Furthermore, although the occluded efforts were relatively easy to perform (as intended by limiting the peak desired negative intrathoracic pressures to only -35 mmHg) many individuals reported that repetitively performing the occluded efforts was a primary factor that contributed to their tiredness. Participants also complained of boredom and they often became restless towards the end of each protocol. Although they were able to watch television, this activity was continuously interrupted due to the frequent requirement for participants to focus on the LED display system and failed to provide an efficient means of distraction.

2.3 Echocardiography

The use of ultrasound to non-invasively generate two-dimensional images of numerous organs has made it an essential tool in the hands of both radiologists and researchers. One of the earliest uses of ultrasound was echocardiography whereby cardiac morphology could be examined. Furthermore, utilising the principles of the Doppler effect, echocardiography is used to measure blood flow in various vessels in the body and additionally across valves of the heart allowing

estimates of systolic pulmonary arterial pressure (SPAP) and cardiac output (\dot{Q}) to be determined.

2.3.1 Two-dimensional cardiac imaging

Two-dimensional images of the heart and other organs are most commonly obtained using a transducer that utilises the principles of the piezoelectricity. When an electric voltage is applied to piezoelectric crystals within the transducer, rapid vibrations from these crystals cause high frequency pressure waves (sound waves) to be generated which can pass through soft tissues. These piezoelectric crystals are also able to receive returning sound waves that are reflected off various tissues of the body (echoes) and convert them into an electrical signal. This electrical signal can then be converted into a two-dimensional image by a computer. The denser a tissue is, the greater the number of echoes returned to the transducer, the greater the voltage produced and thus the brighter the image is. This allows dense cardiac tissue such as, the chamber walls and valves to be distinguished from the less dense tissue (e.g. skin and blood).

Sound waves from the transducer are sent in brief batches and between each batch of sound waves the transducer receives and processes returning echoes, (listening phase). By increasing the duration between each batch of sound waves the 'listening phase' is increased, allowing echoes from further away from the transducer to be received. Although this can be beneficial by increasing the depth

at which images can be generated, it is offset by a reduction in temporal resolution, an important consideration in a constantly moving organ like the heart. An increased depth also reduces spatial resolution. Because sound waves cannot pass through the ribs echocardiography requires a special transducer that is small enough to fit between the ribs. Sound waves emitted from the transducer spread out in a fan shape allowing a larger image to be produced relative to the transducer head size. As shown in figure 2.5 the greater the distance from the transducer the greater the horizontal distance between scan lines. The ability to distinguish between structures lying side by side horizontally (lateral resolution) is impaired as distance from the transducer is increased because ends of the structures may fall between scan lines. Therefore, when attempting to perform accurate measurements of the width of various structures within the heart, these should be performed vertically along scan lines (axial resolution). (see: *Cardiac views*, for an example of using a vertical rather than horizontal alignment to measure aortic diameter).

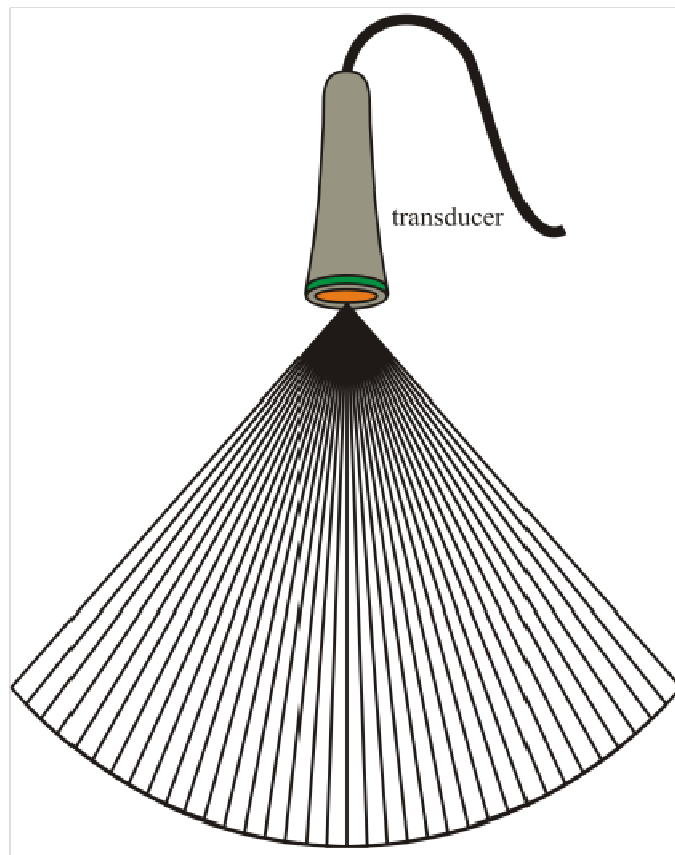


Figure 2.5: Illustration of the scan lines from an ultrasonography transducer.

2.3.2 Doppler ultrasound

Upon generating two-dimensional views of the heart to locate the tricuspid and aortic valves, the principles of the 'Doppler effect' can be utilised to assess the flow of red blood cells across these valves. The "Doppler effect" is named after Christian Johann Doppler who hypothesised that stars appearing red were moving away from the observer and stars appearing blue were moving towards the observer.

as a result of differences in the frequency of their emitted light waves. Three years later Buys Ballot (1845) confirmed that the hypothesis also held true for sound waves. He demonstrated that received sound waves were higher in frequency than those being generated by the sound source as it moved towards him and lower in frequency when it moved away from him.

Based on these principles a brief batch of sound waves of a known frequency is emitted by the ultrasound transducer and measuring the frequency of reflected sound waves from red blood cells allows the velocity of blood to be calculated. If red blood cells are stationary the frequency of returning sound waves will be the same as those first generated by the transducer. The faster the red blood cells are moving towards the transducer, the greater the increase in the frequency of returning sound waves and vice versa for red blood cells moving away from the transducer. Using the mathematical formula shown below the change in frequency of these sound waves can be converted into velocity of the red blood cells (Hatle and Anglesen, 1985) (equation 3),

$$v = \frac{\Delta f C}{2 f \cos \theta} \quad \text{Equation 3}$$

where v is red blood cell velocity, Δf is the change in frequency calculated by the ultrasound machine, θ is the angle between the direction of the red blood cells and the sound wave (assumed to be 0°), and C is the velocity that sound travels in tissue (1540 m/sec).

2.3.3 Doppler modes

Two distinct Doppler modalities exist: pulsed wave Doppler (PWD) which is used to measure blood flow of slow velocities and continuous wave Doppler (CWD) which is used to measure fast velocities. During PWD a batch of sound waves are emitted and the next batch is not generated until the “listening” phase is complete. Increasing the pause between emitting a batch of sound waves and the ‘listening’ phase will increase the distance between the transducer and the point where blood flow velocity is measured. As a compromise of this intermittent pattern of sampling PWD cannot reliably assess high velocity flow (Hatle and Anglesen, 1985). In contrast, CWD can be used for assessing much faster velocities because sound waves are continuously emitted by a segment of the transducer whilst reflected sound waves are continuously recorded by an independent segment. A limitation of CWD is that the absence of pauses between batches of sound waves and the ‘listening’ phase is that the location of echoes cannot be distinguished. It can therefore only be used to calculate peak velocity of blood flow at a desired location if this peak velocity is known to exceed the velocity of blood flow anywhere else along the distance of the scan line (e.g. high velocity flow through valves such as tricuspid regurgitation).

The change in frequency of the reflected sound waves recorded in PWD and CWD are first separated into positive and negative changes representing flow towards and flow away from the transducer. These are then converted into velocity using

the previously mentioned equation (equation 3) and plotted on the y-axis of a graph with time as the x-axis providing a spectral waveform. This is accompanied by an ECG trace to better distinguish the varying phases of the cardiac cycle and respiratory trace so that measurements can be made upon end-expiration (figure 2.6).

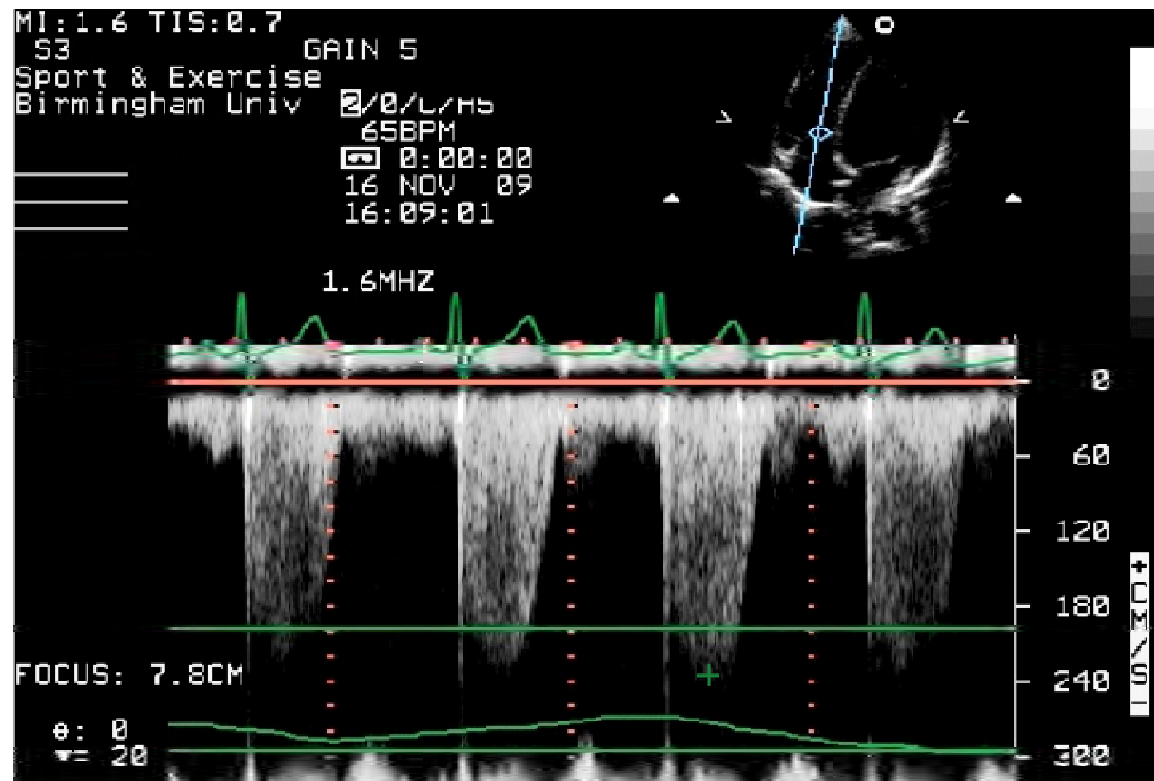


Figure 2.6: A spectral waveform of tricuspid regurgitation measured in continuous wave Doppler mode (CWD). Upper: electrocardiogram; Middle: Doppler waveform (frequency shift converted to positive and negative velocity); Lower: respiratory cycle. Green cross indicates peak velocity of tricuspid regurgitation.

2.3.4 Cardiac views

In this thesis echocardiography was used to assess SPAP and \dot{Q} for which three different cardiac views were required. For the assessment of SPAP the velocity of blood flow across the tricuspid valve was measured using a four-chamber apical view (figure 2.7A). To estimate SV in order to calculate \dot{Q} the velocity of blood flow across the aortic valve was measured using a five chamber apical view (figure 2.7B). Apical views of the heart are performed with the transducer positioned within the fourth or fifth intercostal space and the participant positioned on their left side to allow the apex of the heart to swing closest to the transducer. In order to calculate \dot{Q} , the diameter of the aortic valve needs to be measured as well. Although the aortic valve can be visualised in the apical five-chamber view, measuring its width in this view would be inaccurate as the vessel is parallel to the scan lines and thus the vessel walls may fall between scan lines. For this reason, the aortic valve diameter is measured using a parasternal long axis view where the width can be measured vertically along a scan line (figure 2.7C). To this end, participants lay on their back and the transducer was positioned just lateral to the sternum within the second or third intercostal spaces.

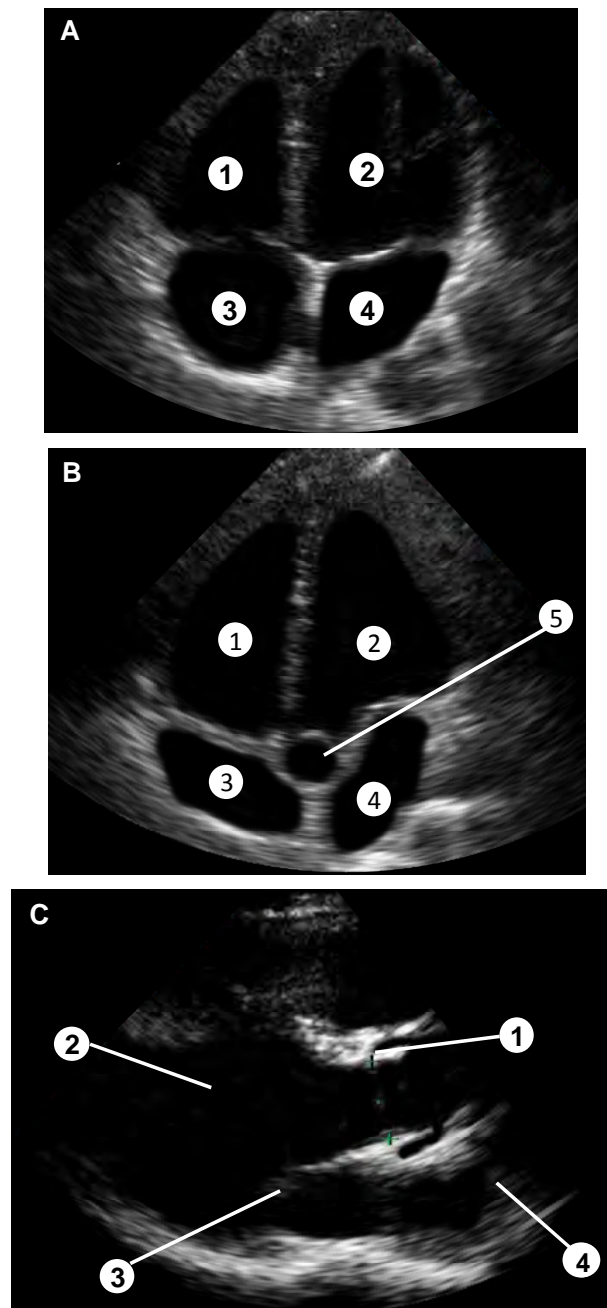


Figure 2.7. (A) Apical four-chamber view: (1) right ventricle, (2) left ventricle, (3) right atrium, (4) left atrium. (B) Apical five-chamber view: (1) right ventricle, (2) left ventricle, (3) right atrium, (4) left atrium, (5) aorta. (C) Parasternal long-axis view: (1) aortic valve, (2) left ventricle, (3) mitral valve, (4) left atrium.

2.3.5 Estimation of SPAP

In 80% of healthy individuals a small degree of regurgitation can be detected across the tricuspid valve during ventricular systole. This trivial volume of regurgitation is not pathological and can be used to indirectly estimate SPAP non-invasively. Tricuspid regurgitation is a fast flowing jet which at its peak velocity is over 200 cm/s thus requiring a high sampling rate to measure the large frequency shift. For that reason Tricuspid regurgitation is measured using CWD in a four-chamber view and identified in colour Doppler mode (figure 2.8).

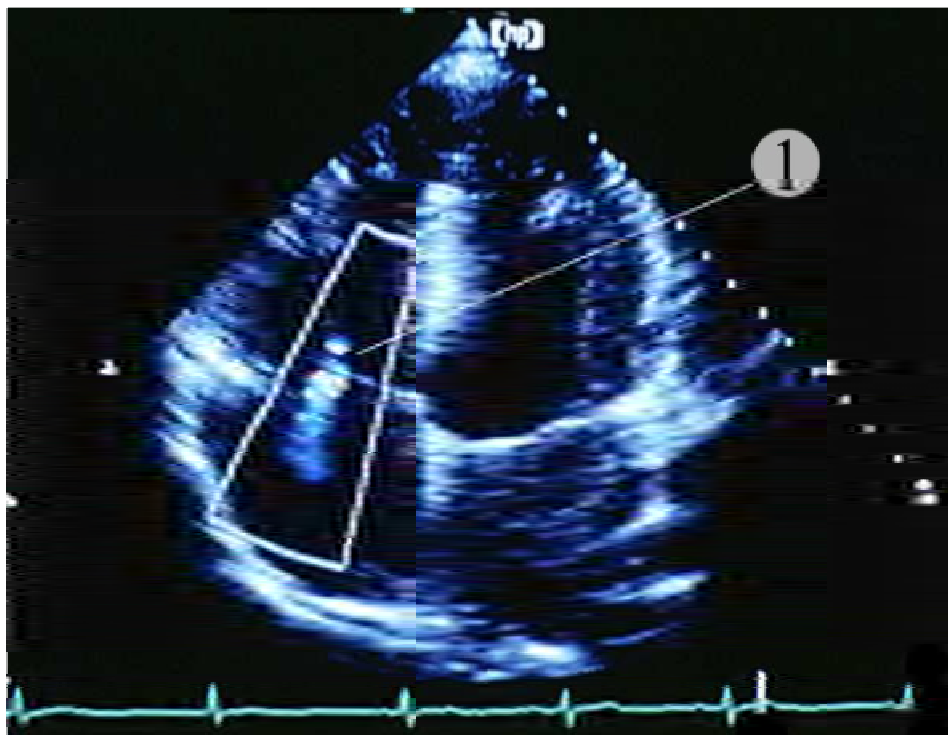


Figure 2.8: Apical 4-chamber view illustrating the tricuspid regurgitation jet (1) as visualised in colour Doppler mode.

Because the velocity of tricuspid regurgitation far exceeds any other flow within the right side of the heart the peak velocity of the spectral trace can always be attributed to tricuspid regurgitation. The peak velocity of the regurgitation jet is measured at the most distal part of the waveform as shown in figure 2.6 and is then converted to a peak pressure drop (ΔP_{max}) between right ventricle pressure and right atrium pressure (RAP) by a modified version of the Bernoulli's equation shown below (equation 4),

$$\Delta P_{max} = 4v^2 \quad \text{Equation 4}$$

where ΔP_{max} is the pressure drop across the orifice in mmHg and v is velocity. To gain accurate measurements with this equation a number of assumptions must be made: firstly, the velocity before the orifice (right ventricle) is low; secondly the orifice is short and flow through it is steady; and thirdly the Doppler scan line is well aligned with the flow. Because right ventricular pressure during systole is equivalent to SPAP the relationship can be depicted as (equation 5),

$$\Delta P_{max} = \text{SPAP} - \text{RAP} \quad \text{Equation 5}$$

RAP is relatively constant at a low value of ~5 mmHg and thus SPAP is assumed to be $\Delta P_{max} + 5$ mmHg.

2.3.6 Cardiac output

Two separate echocardiographic measurements are required for the determination of cardiac output (\dot{Q}). Firstly, a five-chamber view of the heart (figure 2.9) is obtained and the velocity of blood flow across the aortic valve is measured in order

to calculate the velocity time integral (VTI). VTI is essentially the distance that blood travels in the aorta with each ventricular contraction. Secondly, a parasternal view (figure 2.7c) is used to determine the diameter of the aortic orifice, which is assumed to be a circle and thus its area (A) can be calculated using the formula πr^2 .

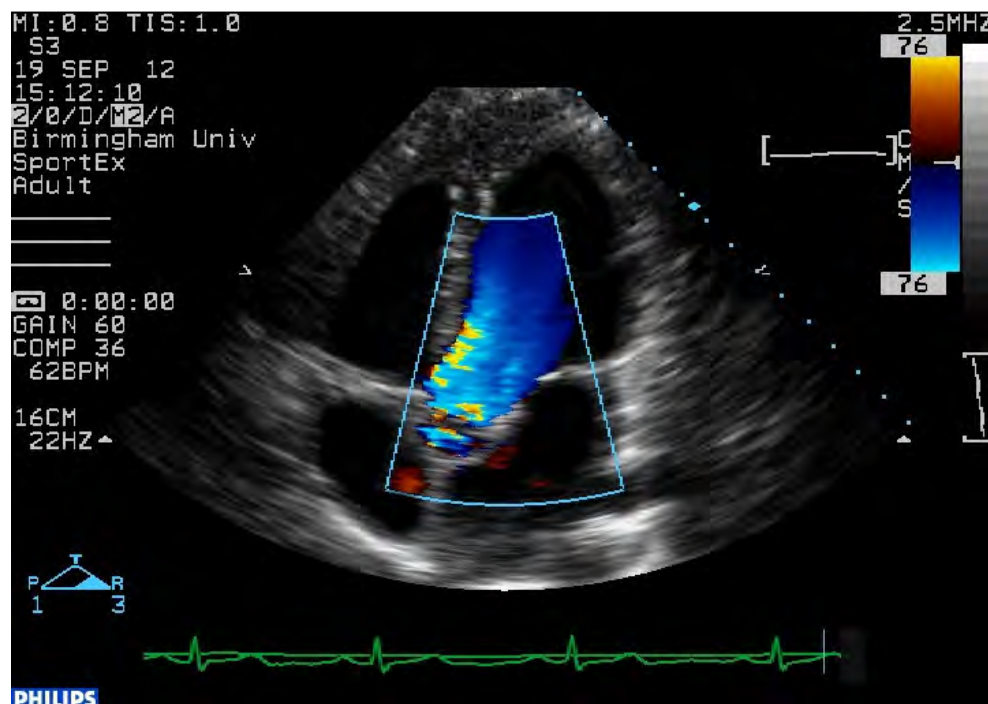


Figure 2.9: Ejection of blood through the aortic valve as visualised in colour Doppler mode using a five-chamber view.

To determine VTI, PWD mode is used to measure the velocity of blood flow through the aortic valve with the gate positioned on the aortic orifice and the Doppler beam aligned with the flow. Mean velocity of the PWD spectral trace is then calculated offline and it is automatically integrated by the ultrasound machine

with the duration of systole to give VTI (figure 2.10). \dot{Q} can then be calculated using the equation below,

$$\dot{Q} = VTI \times A \times HR \quad \text{Equation 5}$$

where HR is heart rate.

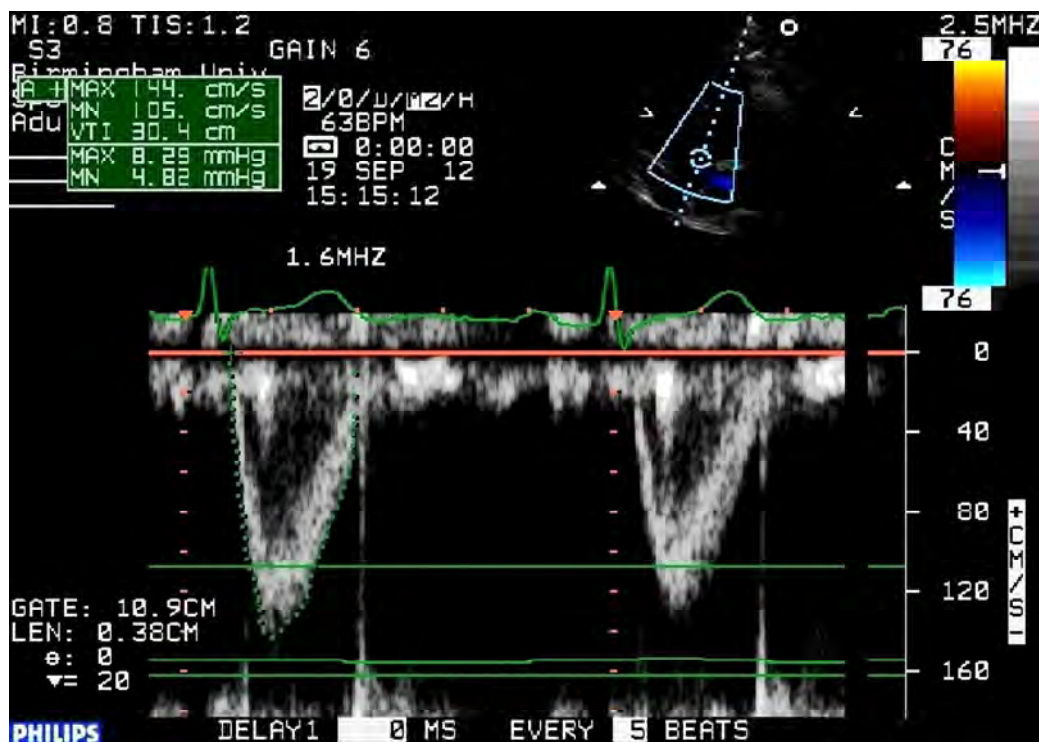


Figure 2.10: A PWD spectral trace of flow through the aortic valve. The mean velocity (VTI) is measured using the green trace.

3 CHAPTER 3 - ASSESSING PULMONARY ARTERIAL PRESSURE DURING AIRWAY OCCLUSIONS USING DOPPLER ULTRASOUND

3.1 Introduction

It is generally accepted that 15-20% of all OSA patients without coexisting pulmonary disorders (e.g. chronic obstructive pulmonary disorder - COPD, obesity hypoventilation disorder etc) suffer from some degree of pulmonary hypertension (Kessler et al., 1996) although a recent study suggested that this may be as high as 20-40% (Sajkov and McEvoy, 2009). Furthermore, numerous studies have demonstrated that OSA is causal in the development of pulmonary hypertension because treatment with CPAP significantly reduces PAP without affecting other factors that are closely associated with OSA, such as excess weight (Sajkov and McEvoy, 2009).

Intravascular PAP which is referenced to atmospheric pressure demonstrates an irregular decline during the time course of an occluded apnoea and returns to baseline levels upon resumption of breathing (Marrone et al., 1989). However, when PAP is expressed as transmural PAP (tPAP) after correcting for the negative intrathoracic pressure swings, it is evident that in contrast there are numerous transient elevations in tPAP throughout an apnoea and also a slower more gradual

elevation that begins mid-apnoea and peaks immediately post apnoea (Marrone et al., 1989, Schafer et al., 1998). It has been hypothesised that these apnoea-induced elevations in tPAP may be responsible for the high prevalence of diurnal pulmonary hypertension in OSA patients (Schroeder et al., 1978). It is plausible that the frequent elevations in tPAP that occur over a relatively long duration (6-8 hours) on a nightly basis may promote vascular remodeling leading to sustained pulmonary hypertension. Indeed, rodents exposed to eight hours of IH per day for four weeks to mimic that experienced in OSA, demonstrate vascular remodeling and developed pulmonary hypertension (Fagan, 2001).

The stimuli responsible for both the numerous rapid transient elevations and also the slow gradual rise in tPAP during occluded apnoeas are not fully understood but two primary mechanisms have been suggested (Marrone et al., 1989, Schafer et al., 1998). Firstly, negative intrathoracic pressure swings associated with occluded inspiratory efforts almost certainly mediate the multiple transient elevations in tPAP (Marrone et al., 1989, Schafer et al., 1998). Each of these rapid increases in tPAP occurs simultaneous to occluded inspiratory efforts and tPAP is correlated to the magnitude of negative pressure. Indeed, this relation is clearly shown in figure 3.1 which is adapted from (Marrone et al., 1989) and shows average tPAP for a range of different negative intrathoracic pressures.

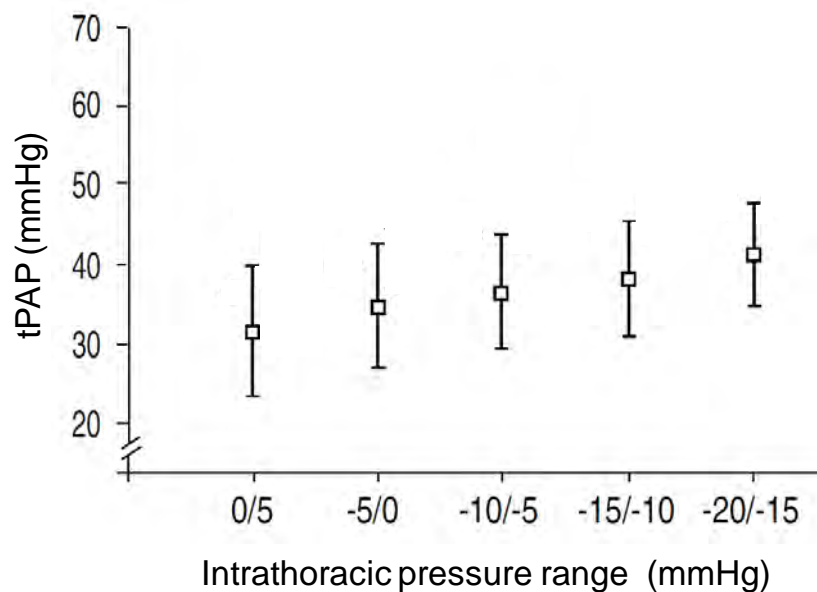


Figure 3.1: Average transmurial PAP (tPAP) over a range of intrathoracic pressures in four patients with OSA. Adapted from (Marrone et al., 1989).

Furthermore, snoring that induces negative intrathoracic pressure swings without a reduction in S_aO_2 elicits these transient elevations in tPAP (Podszus et al., 1991). It is thought that these negative intrathoracic pressures swings may increase tPAP by increasing venous return (Moreno et al., 1967) leading to greater right ventricular output and subsequently elevating pulmonary blood volume. This may occur because blood can more easily flow back towards the heart due to the negative pressure gradient between the extra and intrathoracic regions. In contrast, the positive pressure gradient between intra and extrathoracic regions makes pumping blood away from the heart harder. This may further augment the rise in tPAP because of the simultaneous reduction in SV leading to a greater left

ventricular afterload (Orban et al., 2008, Tolle et al., 1983) and potentially increasing pulmonary wedge pressure.

Secondly, the appearance of hypoxia may cause HPV as studies have shown a significant correlation between the fall in S_aO_2 and the gradual increase in tPAP during occluded apnoeas (Marrone et al., 1989, Marrone et al., 1994, Schafer et al., 1998). Indeed, studies using canine models of OSA provide evidence to support HPV as the responsible mechanism for the gradual elevation in tPAP during occluded apnoeas (Schneider et al., 2000, Iwase et al., 1992). Mild hyperoxic exposure immediately prior to airway occlusions in anaesthetised dogs completely abolished the rise in tPAP in both studies. Furthermore, Schneider et al. (2000) also measured systemic blood pressure and showed hyperoxia caused only a small reduction in the rise in systemic blood pressure. In contrast, blockade of the autonomic nervous system completely abolished the apnoea-induced rise in systemic blood pressure without any attenuation in the rise in tPAP suggesting that a local hypoxia-mediated mechanism is responsible. These results in canines suggesting that the gradual elevation in tPAP during apnoeas in OSA patients is likely to be mediated by HPV fits well with evidence from rodent studies that show chronic exposure to IH independent of other OSA related stimuli induce the development of pulmonary hypertension (Fagan, 2001, McGuire and Bradford, 2001).

As far as we are aware only one study to date has been performed to investigate whether the findings in dogs are also true of humans (Marrone et al., 1992). In this study tPAP was measured continuously via a Swan-Ganz catheter in six sleeping OSA patients. Each patient was exposed to three hours of O₂ supplementation via nasal prongs at a flow rate of 4-6 L/min and three hours room air in a randomly assigned pattern. During both the room air and the hyperoxic phase tPAP was analysed in 15 consecutive apnoeas. In contrast to the findings in dogs, prior O₂ administration did not abolish or even attenuate the rise in tPAP during occluded apnoeas in OSA patients. Taken at face value these results would suggest that the rise in tPAP during OSA is mediated by a hypoxia-independent mechanism but after a thorough critique of the study we have noticed a number of caveats that may account for these unexpected results. Firstly, although the authors report that the prior O₂ supplementation caused a significant attenuation in the decline of S_aO₂, the 4-6 l/min flow of O₂ was insufficient to completely abolish the reduction in S_aO₂, which two participants demonstrated an average nadir of 92%. Furthermore, O₂ supplementation caused the average duration of apnoeas to be lengthened, which presumably accounted for the reported augmentation in the post apnoea level of PETCO₂. When it is considered that S_aO₂ of 92% indicates a significant degree of hypoxia and that hypercapnia is also known to cause pulmonary vasoconstriction (Balanos et al., 2003) it is maybe not surprising that there was still a significant increase in tPAP during occluded apnoeas with prior O₂ administration. Unlike the aforementioned study by (Marrone et al., 1992)), using

our newly developed airway occlusion model of OSA (chapter 2) we are able to clamp PET_{O_2} at an individually tailored, pre-determined mild hyperoxic level immediately prior to airway occlusions to ensure that PET_{O_2} remains in euoxic range (~100 mmHg) following airway occlusions. Furthermore, by using awake participants rather than sleeping OSA patients we are able to control the duration of airway occlusions and ensure an identical duration between hypoxic and mild hyperoxic airway occlusions. Thus, the magnitude of hypercapnia can also be kept similar between hypoxic and mild hyperoxic airway occlusions.

Although there exists a relatively large body of human research investigating the time course of HPV during CH we are unaware of any study to have investigated PAP during exposure to IH that is independent of other stimuli (e.g. those occurring during occluded apnoeas in OSA patients). However, an interesting study on dogs suggests that HPV is sensitised during successive hypoxic exposures (Unger et al., 1977). (Unger et al., 1977) exposed dogs to ten cycles of hypoxia (10% inspired O_2 for 15 minutes) interspersed by euoxia (21% inspired O_2 for 15 minutes) and reported a progressive augmentation in the peak PAP during successive hypoxic exposures. The first hypoxic exposure produced a 28% increase in PAP from euoxic baseline and the tenth produced a 99% increase. Interestingly, PAP returned to baseline levels during each euoxic period that separated hypoxic exposures. This would suggest that HPV was sensitised rather than the vascular tone of pulmonary arteries during euoxia having been reset to a

higher level. In contrast, after an initial rapid increase in PAP following the induction of CH PAP remained stable throughout a further three hours exposure suggesting that in dogs intermittent rather than continuous exposure to hypoxia is required to cause sensitisation of the HPV. Although the duration of hypoxic exposures was substantially greater than those experienced by OSA patients these findings could still have implications for OSA patients exposed to severe IH. However, it should also be noted that in contrast to canines other species such as rabbits demonstrate a substantial secondary HPV phase that lasts several hours during CH (Vejlstrup and Dorrington, 1993). Indeed, it has also been shown in humans that there is also a slow gradual phase that it is not initiated until ~40 minutes (Talbot et al., 2005). Whether sensitisation of the HPV during 15 minute hypoxic exposures as shown in these dogs might also occur during more transient exposures of 15-45 seconds such as those occurring during OSA remains to be determined. Interestingly, in a study of OSA patients who undertook a polysomnography, patients demonstrated a gradual rise in tPAP over the time course of the night (Sforza et al., 1998). In contrast to the results from Unger's (1977) study of dogs that showed a progressive augmentation in PAP during hypoxic episodes without a change in PAP during separating euoxic episodes, tPAP in the OSA patients gradually increased during unrestricted breathing between apnoeas when it was assumed that euoxia had been restored (measured immediately prior to apnoeas) in addition to during hypoxic episodes (measured during occluded apnoeas and immediately afterwards). Because the increase in tPAP occurred not only during the hypoxic apnoeas but also periods of unrestricted

breathing their data would suggest there is a gradual resetting of resting vascular tone rather than an augmentation in HPV. However, the average AHI in OSA patients in this study was 84 which is extremely high when it is considered that 30 or more is used to diagnose severe OSA. The average apnoea length was relatively long at 27 seconds and the average nadir in S_aO_2 was severe at 77.5%. With such a high AHI, long duration of apnoeas and extremely low S_aO_2 nadirs we suspect that patients may have remained relatively hypoxic throughout the night with limited or no complete re-oxygenation phases. The hypoxic stimuli in these patients may thus represent exposure to CH rather than a true intermittent stimulus. If our assumption is correct then the gradual rise in tPAP throughout the time course of the night may better represent the gradual rise in PAP shown in humans during CH that is not initiated until ~40 minutes of sustained hypoxia (Talbot et al., 2005), and takes several hours before it plateaus (Dorrington et al., 1997). We believe that our newly developed airway occlusion model of OSA (chapter 2) might better enable the possibility of sensitisation of the HPV during IH as caused by OSA to be more thoroughly investigated than can be achieved from the data in the study by (Sforza et al., 1998). We are able to evoke significant drops in S_aO_2 following each airway occlusion whilst utilising a lower AHI from that of the OSA patients in Sforza's et al., (1998) study (40 vs. 84) that better resembles the AHI in most OSA patients. We are therefore able to ensure adequate re-oxygenation between airway occlusions that better represents the nightly events of most OSA patients.

PAP is most commonly assessed in a clinical setting using a Swan-Ganz catheter and has been used in all of the previously discussed studies of OSA patients and animals to measure PAP during apnoeas. Although Swan-Ganz catheters are considered the gold standard for assessing PAP as they give accurate absolute values the technique is highly invasive, limiting its use outside of a clinical setting. Assessment of SPAP in a research setting is increasingly made non-invasively using Doppler ultrasound. Although it cannot be claimed that Doppler ultrasound provides extremely accurate absolute values of SPAP it has been shown to provide reproducible values allowing changes in SPAP over time to be assessed (Talbot et al., 2005). Despite a substantial body of research having been performed using Doppler ultrasound to measure SPAP during exposure to CH we are not aware of any research using Doppler ultrasound to assess changes in SPAP during IH or airway occlusions. If Doppler ultrasound can be successfully used to measure SPAP during airway occlusions, future studies undertaken to develop our understanding of the effects of OSA on the pulmonary circulation could be more easily performed.

Study aims

Having successfully developed a new airway occlusion model of OSA as described previously (chapter 2) we planned to use this model in conjunction with Doppler ultrasound in order to investigate the effects of IH on pulmonary haemodynamics. The primary aim of this study was to measure SPAP during airway occlusions with and without hypoxia and we hypothesised SPAP would only rise during hypoxic

apnoeas as previously shown in animal models of OSA (Schneider et al., 2000, Iwase et al., 1992) but incompletely investigated in humans (Marrone et al., 1992). A secondary aim of the study was to investigate whether there is an augmentation in the rise in PAP during airway occlusions over time and to determine whether this is mediated by sensitisation of HPV. We hypothesised that peak SPAP during hypoxic airway occlusions would progressively increase over time but that there would be no change in SPAP during airway occlusions without hypoxia. Furthermore, we wished to examine SPAP during euoxic baseline and during euoxic recovery from airway occlusions with and without hypoxia to assess whether SPAP during euoxia was altered by exposure to repetitive hypoxic apnoeas (i.e. a resetting of resting vascular tone).

3.2 Methods

Ethical approval

After receiving detailed information on the procedures and risks of the study, participants gave written consent to take part. The study was performed according to the latest revision of the Declaration of Helsinki and was approved by the local ethics committee (University of Birmingham ethical review committee).

Participants

Five participants in whom a clear tricuspid regurgitation jet was easily detectable with Doppler ultrasound imaging were recruited to take part in the study. All participants were non-smokers, had no history of cardiovascular, respiratory or metabolic disease and were not taking any medication.

Study protocol

Participants visited the laboratory for two experimental trials in a randomised order separated by at least 24 hours. In one visit participants were exposed to one hour of hypoxic airway occlusions (*Trial 1*) and in the other they were exposed to one hour of mild hyperoxic airway occlusions (*Trial 2*). Prior to experimental visits participants also undertook a preliminary visit to familiarise themselves with the instrumentation and exposure to airway occlusions. For all experimental trials participants were asked to refrain from alcohol consumption and moderate to vigorous exercise for 24 hours and caffeine intake for 12 hours prior to arriving at the laboratory. In addition, all experimental trials were performed at least three hours after food consumption. All trials were performed at the same time of day and were performed in a randomised order.

Preliminary visit

Participants were instrumented in an identical way to both experimental visits. To measure ventilation, induce airway occlusions and control $P_{ET}O_2$ and $P_{ET}CO_2$ for

brief periods prior to airway occlusions, participants breathed through a mouthpiece whilst wearing a nose clip. The mouthpiece was directly connected to a balloon valve (Hans Rudolph, 9340 series, USA) used to occlude the airway, which was in turn connected to a pneumotach (Pneumo Hans Rudolph pneumotach amplifier 1, Shawnee, USA) and turbine (Interface Associates, Irvine, CA, USA) to assess flow and volume, respectively. End-tidal gas was sampled continuously from a catheter placed immediately after the balloon valve and it was analysed using a mass spectrometer (AirSpec 2000, Case Scientific, London, UK). End-tidal profiles were generated using a computerised dynamic end-tidal forcing system, with end-tidal gas composition recorded for each breath and compared with desired values by a computer-controlled, fast gas-mixing system as described previously (chapter 2). Inspired gases were heated and humidified. Respiratory volumes were measured by the turbine and ventilation is reported in BTPS. Pressure at the mouth was continuously assessed with a pressure transducer positioned between the balloon valve and the mouth and used as an index of intrathoracic pressure. Throughout all trials S_aO_2 was continuously measured using a pulse oximeter, worn on the participant's ear lobe (Datex-Ohmeda 3900) and heart rate (HR) was continuously assessed via ECG.

After becoming accustomed to breathing through the mouthpiece participants were exposed to a number of airway occlusions and coached to successfully perform occluded inspiratory efforts as previously described in detail (chapter 2). In brief, during each airway occlusion the balloon valve was rapidly inflated in the breathing

circuit at the end of expiration and remained inflated for 20 seconds. During these 20 seconds participants performed five occluded inspiratory efforts separated by gradually shortening intervals (i.e. inspiratory efforts increased in frequency during the 20-second airway occlusion) with each inspiration requiring increasing effort to generate the desired peak negative intrathoracic pressures of -15, -20, -25, -30 and -35 mmHg. Prior to each airway occlusion participants were briefly exposed to mild hypoxia or mild hyperoxia. PET_{O_2} was clamped at a variety of hypoxic and mild hyperoxic values to identify the desired level of PET_{O_2} for each participant required to produce a post airway occlusion PET_{O_2} nadir of 50 mmHg to be used in *Trial 1* or 100 mmHg to be used in *Trial 2*. During these brief hypoxic and hyperoxic exposures PET_{CO_2} was maintained ~1 mmHg above each participant's normal level.

Experimental trials

Following instrumentation that was identical to that described above for the preliminary visit, participants positioned themselves on a couch in the left lateral position so that Doppler ultrasound measurements could be made when required. Participants then breathed room air for approximately ten minutes to establish stable resting cardiovascular and respiratory parameters before baseline measurements were recorded. Following completion of baseline measurements, in *Trial 1* participants were then exposed to forty 20-second airway occlusions each separated by 70 seconds unrestricted breathing resulting in an AHI of 40. Each

airway occlusion was preceded by 20 seconds of mild hypoxia at the individually tailored PET_{O_2} previously determined in the preliminary visit, whilst PET_{CO_2} was maintained ~ 1 mmHg above baseline levels. In *Trial 2* participants were exposed to forty 20-second airway occlusions again with 75 seconds unrestricted breathing separating them. However, in contrast each airway occlusions was preceded by 15 seconds of mild hyperoxia at the individually tailored PET_{O_2} previously determined in the preliminary visit, whilst PET_{CO_2} was also maintained ~ 1 mmHg above baseline levels. Following completion of the forty airway occlusions (one hour) in both trials participants breathed room air during a 15-minute recovery period.

Doppler ultrasound assessment of SPAP and cardiac output

SPAP and cardiac output (\dot{Q}) were assessed using a Philips Sonos 5500 ultrasound machine with an S3 two-dimensional transducer (1-3 MHz) as previously described in detail (chapter 2). In brief, for assessment of SPAP the tricuspid regurgitation jet was visualised using a four-chamber view and using continuous wave Doppler mode a spectral trace of the jet's velocity profile was recorded. SPAP was estimated offline at a later date from the peak velocity of the tricuspid regurgitation jet. Once several clear spectral waveforms of the tricuspid regurgitation jet had been recorded the transducer was quickly repositioned to allow a five-chamber view of the heart. Pulse wave Doppler mode was then used to generate a spectral trace of the velocity of blood flow through the aortic valve at the level of the aortic orifice. The mean velocity over the course of each cardiac

cycle was calculated offline at a later date and in simple terms it represented the distance that the blood travelled in the aorta, termed velocity time integral (VTI). SV could then be calculated using the formula $VTI \cdot \pi r^2$ where r is the radius of the aortic orifice that was measured at the start of each experimental trial. \dot{Q} could finally be calculated by the formula $\dot{Q} = SV \cdot HR$ where HR was taken from the recorded ECG trace.

To investigate the contribution of hypoxia in any elevation in SPAP during airway occlusions and determine whether there is a progressive augmentation in the increase in SPAP during airway occlusions over time we aimed to make several measurements of SPAP throughout an apnoea cycle. This included making a number of measurements immediately prior to airway occlusions, throughout airway occlusions and for a short period following airway occlusions. We hoped that this would allow generation of a clear time line of changes in SPAP during airway occlusions with and without hypoxia. Measuring \dot{Q} and SPAP require a change in transducer placement and thus could not be performed during the same airway occlusion. Thus, we aimed to assess SPAP and \dot{Q} during alternating airway occlusions. To examine whether there was a change in the response of SPAP to hypoxic and/or mild hyperoxic airway occlusions over the course of one hour we aimed to perform these measurements during a number of airway occlusions at the start, middle and end of the one hour exposures. To determine whether SPAP would remain elevated above euoxic baseline levels following one hour of hypoxic

and/or mild hyperoxic airway occlusions, SPAP and \dot{Q} were assessed at euoxic baseline and during euoxic recovery. To this end, SPAP and \dot{Q} were assessed during the final two minutes of room air breathing prior to airway occlusions and the final two minutes of each five minute period of the fifteen minute room air recovery period in both trials.

Statistical analysis

A two-way analysis of variance (ANOVA) with repeated measures was used to assess whether there was time-by-trial interaction for each haemodynamic measurement (SPAP, HR, SV and \dot{Q}). One-way ANOVA with repeated measures was also performed separately for each trial to better determine the effect of time on these haemodynamic measurements. Values are expressed as mean \pm standard error and differences were considered significant if $P \leq 0.05$.

3.3 Results

Participants

Four male and one female participant completed the experiment with an average age of 23.6 ± 1.9 years, weight of 72.4 ± 6.3 kg, height of 176.4 ± 5.7 cm and a BMI of 23.2 ± 0.89 . All values are mean \pm standard deviation.

Haemodynamic measurements during airway occlusions

Our primary aim was to assess SPAP during airway occlusions that closely mimicked occluded apnoeas in OSA with or without hypoxia and thus determine the contribution of hypoxia to any elevation in SPAP. The modest reduction in PET_{O_2} prior to each airway occlusion in *Trial 1* was successful in eliciting a significant degree of hypoxia post airway occlusion as PET_{O_2} dropped to an average nadir of 50.57 ± 0.51 mmHg. However, we quickly realised that it was not feasible to perform Doppler ultrasound measurements of SPAP during airway occlusions with continued inspiratory efforts. This was because assessment of SPAP requires a high level of transducer control that was compromised by substantial rib cage movement during each occluded inspiratory effort. Therefore, participants were asked not to perform occluded efforts during a number of airway occlusions at the start, middle and end of the hour when SPAP and \dot{Q} were to be assessed. However, despite this compromise we were still unable to record reliable measurements of SPAP at regular enough time points over the time course of an airway occlusion. Furthermore, recording SPAP in the first ~5 seconds immediately post airway occlusion was extremely difficult due to substantial hyperventilation and thus considerable rib cage movement. Therefore, we were unable to describe an accurate time course of changes in SPAP during hypoxic or mild hyperoxic airway occlusions.

Haemodynamic measurements during room air breathing

In contrast to the difficulty in attaining haemodynamic measurements using Doppler ultrasound during airway occlusions, clear and reliable measurements were made during unrestricted room air breathing at baseline and recovery. Figure 3.2 shows SPAP at baseline and during 5, 10 and 15 minutes of recovery following one hour of hypoxic airway occlusions (*Trial 1*) or mild hyperoxic airway occlusions (*Trial 2*). SPAP did not change from baseline to recovery in either trial (*Trial 1*: $P = 0.792$; *Trial 2*: $P = 0.746$). Figure 3.3 shows \dot{Q} at baseline and during 5, 10 and 15 minutes of recovery following one hour of hypoxic airway occlusions (*Trial 1*) and mild hyperoxic airway occlusions (*Trial 2*). As was the case for SPAP, there was no notable change in \dot{Q} from baseline to recovery (*Trial 1*: $P = 0.428$; *Trial 2*: $P = 0.747$). Figure 3.4 and 3.5 show that HR and SV did not change from baseline to recovery in either trial (*Trial 1*: $P = 0.419$; *Trial 2*: $P = 0.608$ and *Trial 1*: $P = 0.940$; *Trial 2*: $P = 0.667$ respectively).

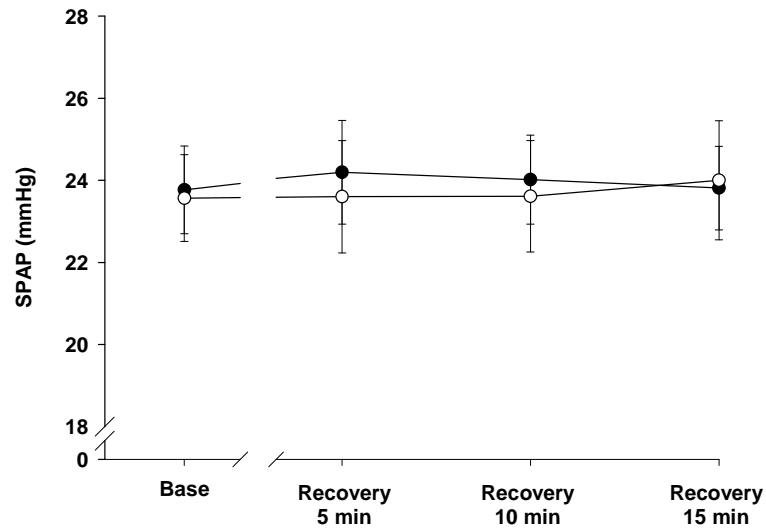


Figure 3.2: Systolic pulmonary arterial pressure (SPAP) at baseline, 5, 10, and 15 minutes of recovery, following one hour of airway occlusions with (closed symbols) and without hypoxia (open symbols).

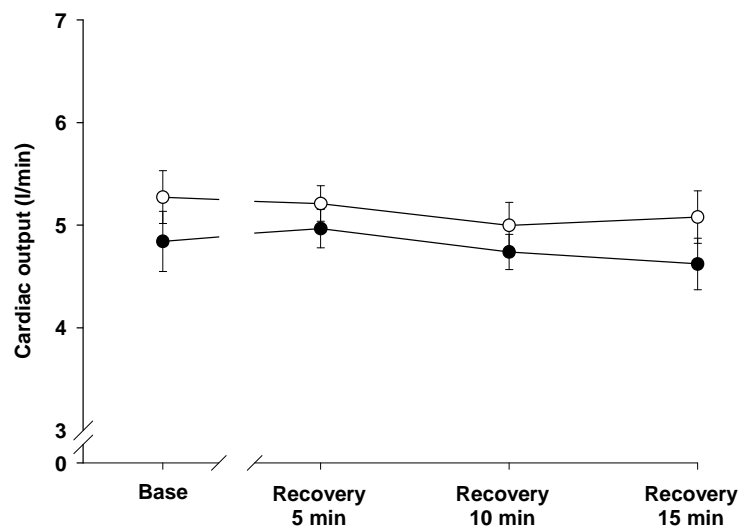


Figure 3.3: Cardiac output at baseline, 5, 10, and 15 minutes of recovery, following one hour of airway occlusions with (closed symbols) and without hypoxia (open symbols).

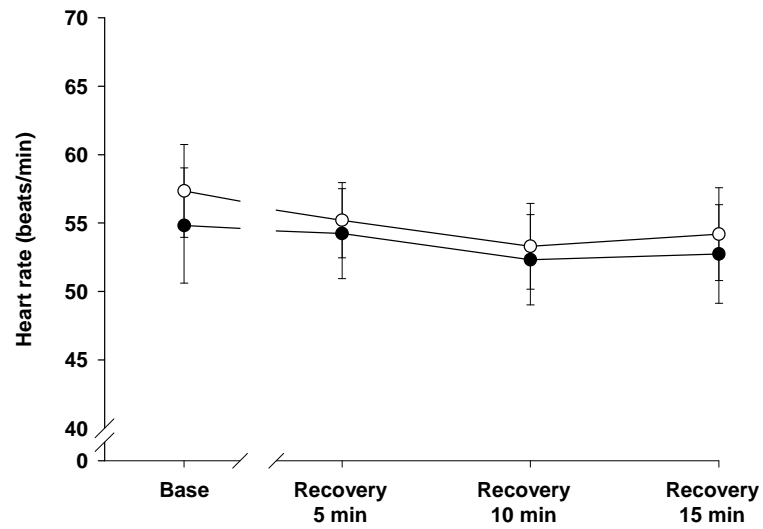


Figure 3.4: Heart rate (HR) at baseline, 5, 10, and 15 minutes of recovery, following one hour of airway occlusions with (closed symbols) and without hypoxia (open symbols).

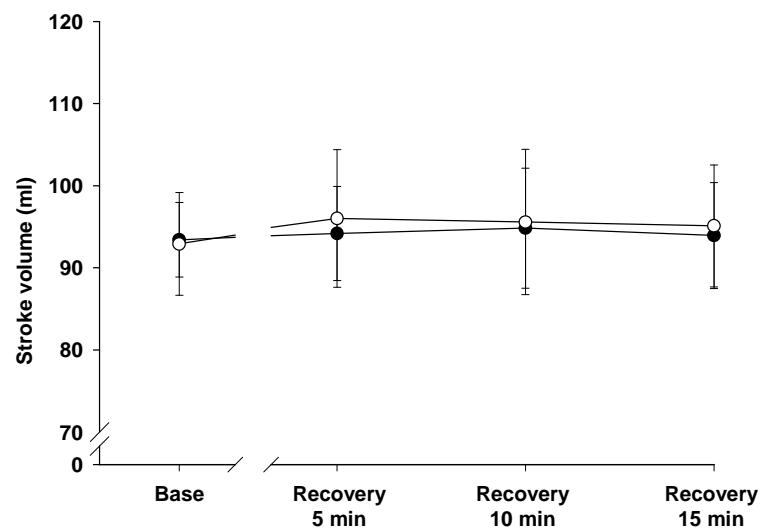


Figure 3.5: Stroke volume (SV) at baseline, 5, 10, and 15 minutes of recovery, following one hour of airway occlusions with (closed symbols) and without hypoxia (open symbols).

3.4 Discussion

With the prevalence of pulmonary hypertension being high in the OSA population (Kessler et al., 1996, Sajkov and McEvoy, 2009) and with recent evidence demonstrating that OSA is causal in its development (Sajkov and McEvoy, 2009) it is of paramount importance that the responsible mechanisms are determined. Only then can therapeutic strategies be developed to prevent its development and progression in OSA patients. It has long been known that hypoxia causes vasoconstriction of the pulmonary vasculature and that chronic HPV can cause pulmonary hypertension in a range of pathophysiological conditions (COPD, obesity hypoventilation disorder etc) and indeed in rats exposed to chronic IH (Fagan, 2001, McGuire and Bradford, 2001). Therefore, it is logical that researchers have attempted to examine the possibility that hypoxia mediates the rise in PAP that occurs during apnoeas in OSA patients. All published studies to date that have measured PAP during obstructive apnoeas have used Swan-Ganz catheters. Although this technique is considered the gold standard for assessing PAP it is highly invasive. Indeed, the relatively limited number of studies undertaken to examine the effects of OSA and specifically IH on pulmonary haemodynamics is no doubt predominantly a result of the invasive nature of this technique. However, in recent years Doppler ultrasound has been increasingly used as an alternative non-invasive approach to estimate SPAP and produces comparable results to Swan-Ganz catheters when assessing changes in SPAP

over time (Talbot et al., 2005). Therefore, we attempted to use this technique to determine the contribution of hypoxia in the rise in PAP during airway occlusions that previous studies of OSA patients have been unable to answer. In doing so we also hoped we would demonstrate the potential use of Doppler ultrasound in future research into the effects of IH in humans.

Prior to undertaking this study we were aware that it would be extremely challenging to accurately measure SPAP during airway occlusions. We screened a large number of volunteers and we recruited only participants with a clear tricuspid regurgitation jet that could be recorded quickly and consistently. However, having attempted to assess changes in SPAP during airway occlusions in awake humans we have had to conclude that Doppler ultrasound is not a viable technique to make these measurements. Consequently, we have been unable to assess what is the contribution of hypoxia to the rise in SPAP during airway occlusions or whether there is an augmentation in this rise in SPAP during a prolonged exposure to repetitive airway occlusions. The most challenging period to measure SPAP was immediately following the airway occlusion when participants hyperventilated heavily. This was important as SPAP is known to gradually increase during apnoeas, starting at the middle of the apnoea and reaching a maximum several breaths post apnoea (Podszus et al., 1994).

During unrestrained room air breathing at baseline and recovery we were able to take accurate measurements of SPAP. Although, we are tentative to draw

conclusions based on such a small study population, none of the five participants involved in the study demonstrated any notable elevation in SPAP during the fifteen minutes recovery following hypoxic or mild hyperoxic airway occlusions. (Sforza et al., 1998) previously demonstrated that PAP gradually increased during the night over the entire apnoea cycle (i.e pre, during and post apnoeas). Because PAP increased during pre apnoea periods it would suggest that PAP was greater during periods of euoxia. However, the AHI was very high, apnoeas were long and average S_aO_2 nadirs were very low, thus we speculated that euoxia may not have been restored during the brief intervals between apnoeas. The gradual rise in PAP during these pre-apnoea periods may better represent the gradual elevation in HPV magnitude seen during CH as previously shown in humans (Talbot et al., 2005, Dorrington et al., 1997) rather than an increase in PAP under euoxic conditions. In our study, hypoxic airway occlusions were limited to just one hour in our study based on previous testing of the model showing that it was too uncomfortable to maintain for longer. It remains to be determined whether a longer exposure to the hypoxic airway occlusions such as the 6-8 hours that occur during sleep in OSA patients might cause a notable sustained elevation in PAP under euoxic conditions.

Another limitation to using Doppler ultrasound in this study was that participants complained of being uncomfortable due to having to remain on the couch positioned in the left lateral position for the entire protocol (~90 minutes). It is possible that participant discomfort could confound results such as systemic BP,

HR and ventilation. This therefore needs to be considered in the design of all future studies using this airway occlusion model.

Summary

Although Doppler ultrasound can be used to investigate changes in SPAP during unrestricted breathing it is not a suitable tool to assess changes in SPAP during airway occlusions. Therefore, we were unable to determine what contribution hypoxia may have on elevated SPAP during airway occlusions and whether there is an augmentation in this response over a prolonged period of airway occlusions. In the small study population SPAP was not elevated above euoxic baseline levels during fifteen minutes of euoxic recovery following airway occlusions with or without hypoxia.

4 CHAPTER 4 - MANIFESTATION OF RESPIRATORY PLASTICITY FOLLOWING HYPOXIC AIRWAY OCCLUSIONS IN AWAKE HUMANS

4.1 Introduction

LTF of ventilation is a sustained elevation in \dot{V}_E that is initiated upon resumption of normal inspired O_2 levels following acute IH. An additional form of respiratory plasticity evoked by IH is a progressive augmentation of the HVR. Both forms of respiratory plasticity have attracted a significant amount of interest primarily because of the possible implications they may have on upper airway stability in OSA patients as previously described in detail (General introduction: *Could OSA-induced IH evoke respiratory plasticity and thus attenuate upper airway collapse?*).

Although airway obstructions in OSA evoke a significant magnitude of IH that would be expected to evoke respiratory plasticity, to date there exists no conclusive evidence demonstrating the manifestation of ventilatory LTF and/or progressive augmentation of the HVR. Indeed, OSA severity during the night does not appear to reduce as might be expected if ventilatory LTF had developed. However, this can't be taken as conclusive evidence for the absence of ventilatory LTF as the development of progressive augmentation of the HVR might negate any benefit of ventilatory LTF (Mateika and Narwani, 2009). The assessment of

whether respiratory plasticity manifests during a night's sleep in OSA or not, is complicated by the absence of suitable intervals of unrestricted breathing to assess normal ventilation. Additionally, the fact that changes in sleep stages induce rapid changes in ventilation may confound this assessment further. An alternative approach is to model OSA in awake humans where these variables can be better controlled. Indeed, a number of studies have exposed awake humans to an acute bout of repetitive hypoxic breath-holds to mimic OSA (Cutler et al., 2004b, Cutler et al., 2004a, Leuenberger et al., 2007, Leuenberger et al., 2005) and have measured ventilation. Ventilation during a prolonged room air recovery period following exposure to hypoxic breath-holds has been compared to baseline room air breathing but none of these studies were able to demonstrate any sustained elevation in ventilation. Furthermore, in one study participants were exposed to five minutes of poikilcapnic hypoxia before and after hypoxic breath-holds but also failed to demonstrate any augmentation in the HVR (Leuenberger et al., 2007). However, an important factor to consider in these studies is that ventilation was assessed at baseline and during recovery from the hypoxic breath-holds under poikilcapnic conditions. During wakefulness, ventilation can be sustained by arousal and/or behavioural stimuli even when PaCO_2 levels are below the ventilatory threshold. Thus under poikilcapnic conditions in these studies the responsible mechanism(s) for mediating respiratory LTF may have been activated during exposure to the repetitive hypoxic breath-holds but the expression of respiratory LTF may have been restrained because ventilation was not controlled

by the chemoreflex. Indeed, (Harris et al., 2006) exposed participants to intermittent reductions in $P_{I_{O_2}}$ and demonstrated the appearance of ventilatory LTF and progressive augmentation of the HVR when $P_{ET_{CO_2}}$ was raised by 5 mmHg above normal levels but not during poikilocapnic conditions.

Using our newly developed airway occlusion model of OSA (chapter 2) in awake humans, we aim to further investigate whether respiratory plasticity might be evoked in OSA. Following exposure to the hypoxic airway occlusions a recovery period of room air breathing will allow results to be compared to the previous hypoxic breath-hold studies. In addition, following room air recovery, raising $P_{ET_{CO_2}}$ slightly above normal levels will allow the possible manifestation of ventilatory LTF when ventilation is driven by the chemoreflex to be investigated. Furthermore, by maintaining the $P_{ET_{CO_2}}$ slightly above normal levels during a brief hypoxic exposure it may be possible to determine whether progressive augmentation of the HVR can also be expressed.

Although we were unable to use Doppler ultrasound to assess the contribution of hypoxia to changes in SPAP during airway occlusions this technique has previously been used to accurately measure the magnitude and the time course of HPV during longer hypoxic exposures (Talbot et al., 2005). Using a similar protocol would allow us to investigate whether HPV is augmented following exposure to hypoxic airway occlusions. Although this would not give any indication of the

intensity of HPV during airway occlusions, showing that HPV is sensitised following a period of hypoxic airway occlusions could have serious implications for the high number of OSA patients with coexisting respiratory conditions such as chronic obstructive pulmonary disease (COPD) (Owens and Malhotra, 2010).

Study aims

The primary aim of this study was to determine whether exposure to an acute bout of hypoxic airway occlusions is capable of evoking ventilatory LTF and/or a progressive augmentation of the HVR in awake humans. A secondary aim of this study was to assess whether HPV during a prolonged hypoxic exposure is augmented by prior exposure to an acute bout of hypoxic airway occlusions.

4.2 Methods

Ethical approval

After receiving detailed information on the procedures and risks of the study participants gave written consent to take part. The study was performed according to the latest revision of the Declaration of Helsinki and was approved by the local ethics committee (University of Birmingham ethical review committee).

Participants

Eleven healthy participants were recruited to take part in the study. In five of these participants a clear tricuspid regurgitation jet could be visualised and thus additional Doppler ultrasound measurements during hypoxic exposures were possible. All participants were non-smokers, had no history of cardiovascular, respiratory or metabolic disease and were not taking any medication.

Study protocol

Participants visited the laboratory for two experimental trials separated by at least 24 hours. In one visit participants were exposed to one hour of hypoxic airway occlusions (*Trial 1*) and in the other they were exposed to one hour of unrestricted room air breathing (*Trial 2*). Prior to experimental visits participants also undertook a preliminary visit to firstly assess whether tricuspid regurgitation could be measured and to familiarise themselves with the instrumentation and exposure to airway occlusions. For all experimental trials participants were asked to refrain from alcohol consumption and moderate to vigorous exercise for 24 hours and caffeine intake for 12 hours prior to arriving at the laboratory. In addition all experimental trials were performed at least 3 hours after food consumption. Both trials were performed at the same time of day and were performed in a randomised order.

Preliminary visit

The preliminary visit involved the exact same procedures as previously described in chapter 3 except for one change. As this study did not involve a hyperoxic airway occlusion trial, during the preliminary visit participants were only exposed to hypoxic airway occlusions.

Experimental trials

In both experimental trials instrumentation was identical to that of the preliminary visit (see: chapter 3). Participants then breathed room air for approximately 15 minutes to establish stable resting cardiovascular and respiratory parameters. Participants were then exposed to forty 20-second hypoxic airway occlusions in *Trial 1* lasting a total of one hour as previously described in chapter 3. In contrast, in *Trial 2* participants breathed room air freely for one hour. Following hypoxic airway occlusions (*Trial 1*) or unrestricted room air breathing (*Trial 2*) participants breathed room air during a ten-minute recovery period.

In both trials participants were then exposed to an acute hypoxic ventilatory response test (AHVR) with the five participants who were undergoing Doppler ultrasound measurements re-positioning themselves into a left lateral position. For the AHVR test, participants were first exposed to five minutes of isocapnic euoxia ($PET_{O_2} = 100$ mmHg and $PET_{CO_2} \sim 1$ mmHg above normal), followed by 20 minutes

of isocapnic hypoxia ($P_{ETO_2} = 50$ mmHg and $P_{ETCO_2} \sim 1$ mmHg above normal) and finally a further five minutes of isocapnic euoxia.

Ventilatory analysis

In both trials, \dot{V}_E , P_{ETCO_2} , P_{ETO_2} and S_aO_2 were recorded continuously. In both trials, baseline measurements were achieved by averaging the last three minutes of the room air baseline period. Likewise, in both trials, measurements were averaged during the final three minutes of the first and second five minutes of the ten minute room air recovery period that followed one hour of hypoxic apnoeas (*Trial 1*) or one hour unrestricted room air breathing (*Trial 2*). In addition, measurements during the AHVR test were achieved by averaging the final three minutes of the five minute isocapnic euoxic baseline period, of each of the five minute quarters of isocapnic hypoxia and of the final five minutes of isocapnic euoxic recovery.

Haemodynamic analysis

In the five participants whom Doppler ultrasound measurements were made SPAP and \dot{Q} was assessed as previously described in detail in chapter 3. In order to measure both SPAP and \dot{Q} during the AHVR test it was necessary to quickly switch between the two different echocardiographic views required (i.e. four and five chamber views respectively). This was achieved by holding a four chamber view of the heart for assessment of tricuspid regurgitation for ~90 seconds allowing

SPAP measurements during the first half of each minute. After ~90 seconds the view was changed to a five chamber view of the heart for ~30 seconds allowing \dot{Q} assessments for the second half of every two minutes. To determine an isocapnic euoxic baseline value for SPAP, \dot{Q} , HR and SV and a value during isocapnic hypoxia once a plateau had been reached, values were averaged for all five minutes of isocapnic euoxic baseline and the last ten minutes of the isocapnic hypoxic exposure.

Statistical analysis

A two-way ANOVA with repeated measures in conjunction with *post hoc* LSD test was used to assess whether there was an interaction between trial and time for all ventilatory measurements (\dot{V}_E , P_{ETCO_2} , P_{ETO_2} and S_aO_2) under room air conditions (three time points) and also separately during the AHVR test (six time points). One way repeated-measures ANOVA in conjunction with *post hoc* LSD test was also performed separately for each trial to better determine the effect of time on ventilatory measurements. For five of the participants who had Doppler ultrasound assessments a two-way ANOVA with repeated measures was used to assess whether there was an interaction between trial and time for SPAP, \dot{Q} , HR and SV. One way repeated-measures ANOVA was also performed separately for each trial to better determine the effect of time on these variables. Values are expressed as mean \pm standard error and differences were considered significant if $P \leq 0.05$.

4.3 Results

Participants

Eleven healthy male participants completed the experiment with an average age of 23.3 ± 1.7 years, weight of 77.1 ± 9.6 Kg, height of 180.1 ± 8.0 cm and a BMI of 23.7 ± 2.2 . Of these eleven participants the five who were assessed for SPAP had an average age of 23.2 ± 1.8 years, their weight and height was 70.0 ± 6.0 kg and 175.0 ± 4.4 cm and they had a BMI of 22.8 ± 1.2 . All values are mean \pm SD.

Hypoxic airway occlusions

The modest reduction in PET_{O_2} prior to each airway occlusion in *Trial 1* was successful in eliciting a significant degree of hypoxia post airway occlusion as PET_{O_2} dropped to an average nadir of 50.61 ± 0.68 mmHg.

Room air breathing

Table 4.1 lists the average PET_{O_2} , PET_{CO_2} and S_aO_2 during room air baseline and room air recovery in both trials. Analysis showed a significant trial-by-time interaction in PET_{CO_2} ($P = 0.001$). Indeed, PET_{CO_2} significantly decreased from baseline to recovery in *Trial 1* ($P = 0.003$) but not in *Trial 2* and thus PET_{CO_2} was significantly lower in *Trial 1* than *Trial 2* during the end of recovery. There was also a significant trial-by-time interaction in PET_{O_2} ($P = 0.043$) as a result of a small but significant decrease in PET_{O_2} from baseline to recovery in *Trial 1* ($P = 0.014$) but

not in *Trial 2*. Thus, $P_{ET_{O_2}}$ was significantly lower in *Trial 1* than *Trial 2* during early recovery but had returned to a similar value by late recovery.

Figure 4.1 shows \dot{V}_E during room air baseline and room air recovery in both trials. \dot{V}_E demonstrated a small but significant decrease from baseline to recovery in both trials (*Trial 1*: $P = 0.004$; *Trial 2*: $P = 0.008$) that was not significantly different between trials.

AHVR test

Gas control: Figure 4.2 shows the $P_{ET_{O_2}}$ and $P_{ET_{CO_2}}$ that were achieved during every minute of the AHVR test in both trials which were not different between trials. Furthermore, Table 4.2 lists the average $P_{ET_{O_2}}$, $P_{ET_{CO_2}}$ and S_aO_2 for the same time periods that ventilation was averaged over during each of the different phases of the AHVR test (i.e. isocapnic euoxic baseline, each quarter of the isocapnic hypoxic exposure and isocapnic euoxic recovery).

Ventilation: Figure 4.3 shows average \dot{V}_E during all phases of the AHVR test in *Trial 1* and *Trial 2*. As was expected \dot{V}_E during the AHVR test in both trials significantly increased from isocapnic euoxic baseline to isocapnic hypoxia and remained elevated for the entire 20 minute exposure before returning to baseline levels during isocapnic euoxic recovery (*Trial 1*: $P = 0.003$; *Trial 2*: $P = 0.006$). Analysis was unable to demonstrate a significant trial-by-time interaction in \dot{V}_E

during the entire AHVR test but there was a significant overall trial effect ($P = 0.003$) with *post hoc* tests showing \dot{V}_E was greater in *Trial 1* vs. *Trial 2* during isocapnic euoxic baseline and all four quarters of the isocapnic hypoxic exposure but not isocapnic euoxic recovery where there was greater variation.

The greater \dot{V}_E during both baseline isocapnic euoxia and isocapnic hypoxia in *Trial 1* vs. *Trial 2* would suggest the manifestation of ventilatory LTF and progressive augmentation of the HVR following the hypoxic airway occlusions in *Trial 1*. The manifestation of ventilatory LTF was further supported by analysis that showed a significant trial-by-time interaction when \dot{V}_E during room air baseline was compared to isocapnic euoxic baseline of the AHVR test ($P = 0.013$). Indeed, elevating PET_{CO_2} of ~ 1 mmHg above room air baseline levels increased \dot{V}_E significantly more in *Trial 1* than *Trial 2* (*Trial 1*: 3.84 ± 0.94 vs. *Trial 2*: 1.53 ± 0.47 l/min) as shown in figure 4.4.

Haemodynamics

Figure 4.5 shows SPAP for *Trial 1* and *Trial 2* during every minute of the AHVR test. In both trials SPAP significantly changed over time (*Trial 1*: $P = 0.006$, *Trial 2*: $P = 0.001$) but was not different between trials. Indeed, the increase from isocapnic euoxic baseline to a stable value during isocapnic hypoxia was by a similar magnitude in both trials (*Trial 1*: 22.8 ± 0.58 vs. 29.7 ± 0.95 mmHg; *Trial 2*: 22.8 ± 0.83 vs. 29.5 ± 0.90 mmHg). Figure 4.6a shows \dot{Q} for *Trial 1* and *Trial 2* during

every minute of the AHVR test. Both trials demonstrated a significant change over time (*Trial 1*: $P = 0.002$, *Trial 2*: $P = 0.019$) but this was not different between trials as indicated by a similar increase from isocapnic euoxic baseline to a stable value during isocapnic hypoxia (*Trial 1*: 4.23 ± 0.09 vs. 5.00 ± 0.20 l/min; *Trial 2*: 4.29 ± 0.32 vs. 4.98 ± 0.31 l/min). In both trials the change in \dot{Q} was a product of a significant increase in HR (*Trial 1*: $P = 0.001$, *Trial 2*: $P = 0.004$) which was again not different between trials as shown in Figure 4.6b. Indeed, there was a similar increase from isocapnic euoxic baseline to a stable value during isocapnic hypoxia (*Trial 1*, 50.4 ± 1.4 vs. 59.6 ± 3.4 bpm; *Trial 2*, 49.7 ± 2.3 vs. 58.6 ± 4.0 bpm). Figure 4.6c shows SV for both trials which did not change over time in either trial (*Trial 1*, 84.0 ± 1.6 vs. 84.4 ± 1.8 ml; *Trial 2*, 86.6 ± 5.4 vs. 85.7 ± 3.5 ml).

Time	Trial 1			Trial 2		
	Baseline	Rec 5 min	Rec 10 min	Baseline	Rec 5 min	Rec 10 min
PETCO ₂ (mmHg)	40.4 ± 0.7	39.2 ± 0.8 *	39.3 ± 0.7 *†	40.4 ± 0.6	40.2 ± 0.5	40.4 ± 0.5
PETO ₂ (mmHg)	100.2 ± 0.5	95.5 ± 1.4 *†	98.3 ± 0.8 *	101 ± 1.2	99.5 ± 0.9	99.4 ± 0.8
S _a O ₂ (%)	97.2 ± 0.1	96.6 ± 0.2	96.6 ± 0.1	97.1 ± 0.2	96.8 ± 0.2	96.8 ± 0.2

Table 4.1: PETCO₂, PETO₂ and S_aO₂ values during room air baseline and room air recovery following one hour of hypoxic airway occlusions (*Trial 1*) or unrestricted room air breathing (*Trial 2*). * significantly lower than baseline, † significantly lower than the same time point in *Trial 2* ($P \leq 0.05$).

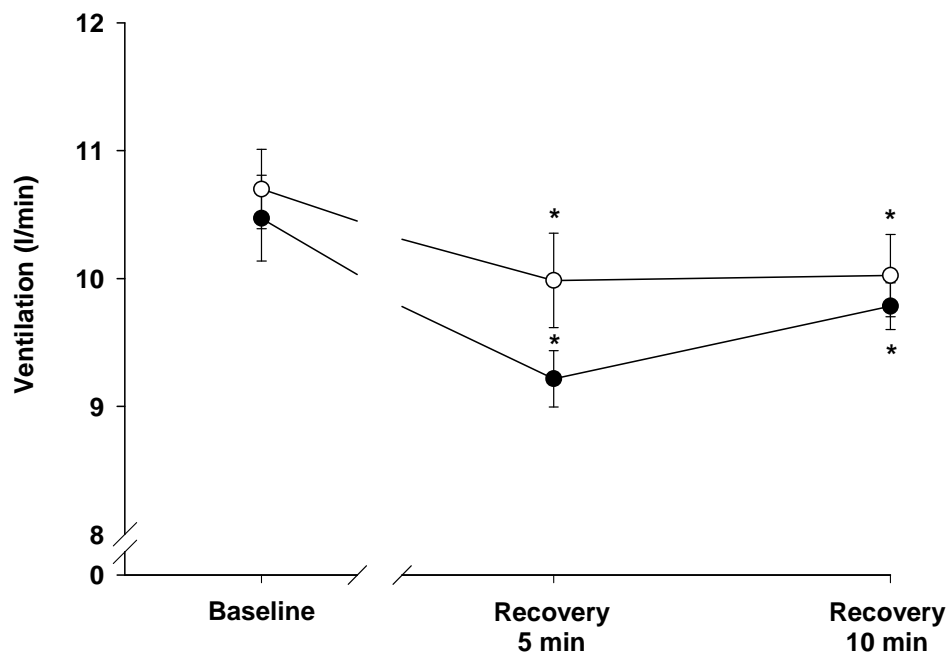


Figure 4.1: Minute ventilation during room air baseline, and room air recovery, following one hour of hypoxic airway occlusions in *Trial 1* (closed symbols) or unrestricted room air breathing in *Trial 2* (open symbols). * significantly lower than baseline ($P \leq 0.05$).

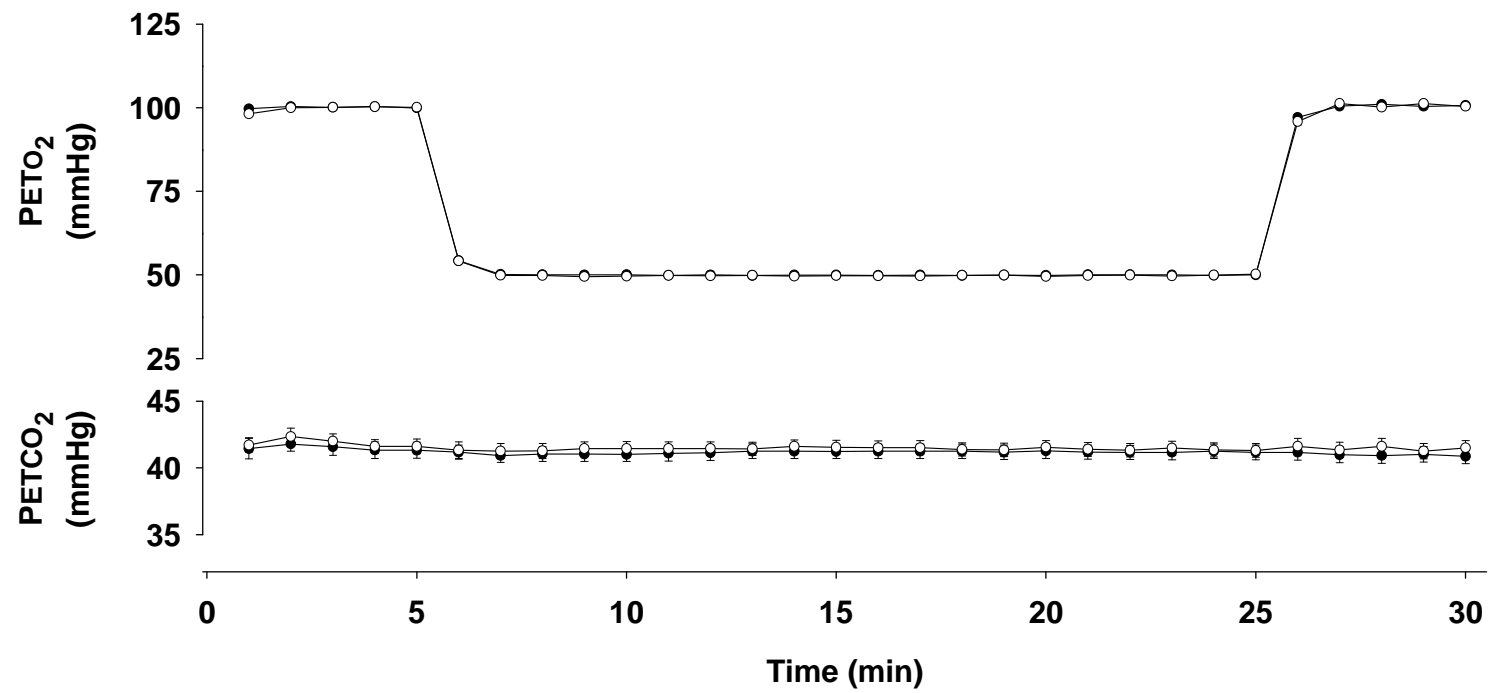


Figure 4.2: PETCO₂ and PETO₂ averaged for every minute during the AHVR test in *Trial 1* (closed symbols) and *Trial 2* (open symbols).

Trial 1						
Time	Baseline	Hypoxia 5 min	Hypoxia 10 min	Hypoxia 15 min	Hypoxia 20 min	Recovery
PETCO ₂ (mmHg)	41.4 ± 0.6	41.0 ± 0.5	41.2 ± 0.5	41.2 ± 0.5	41.2 ± 0.6	40.9 ± 0.6
PETO ₂ (mmHg)	100.1 ± 0.2	50.0 ± 0.1 *	49.9 ± 0.1 *	49.9 ± 0.0 *	50.0 ± 0.0 *	100.7 ± 0.2
S _a O ₂ (%)	96.9 ± 0.2	86.6 ± 0.4 *	86.3 ± 0.5 *	86.3 ± 0.5 *	86.3 ± 0.4 *	96.8 ± 0.1

Trial 2						
Time	Baseline	Rec 5 min	Rec 10 min	Rec 10 min	Rec 10 min	Recovery
PETCO ₂ (mmHg)	41.7 ± 0.5	41.4 ± 0.5	41.5 ± 0.5	41.4 ± 0.5	41.4 ± 0.5	41.4 ± 0.6
PETO ₂ (mmHg)	100.2 ± 0.1	49.6 ± 0.2 *	49.7 ± 0.1 *	49.8 ± 0.1 *	50.0 ± 0.1 *	100.6 ± 0.2
S _a O ₂ (%)	97.0 ± 0.2	86.5 ± 0.4 *	86.3 ± 0.4 *	86.5 ± 0.5 *	86.6 ± 0.4 *	96.9 ± 0.1

Table 4.2: Averaged PETCO₂, PETO₂ and S_aO₂ values for isocapnic euoxic baseline, each quarter of isocapnic hypoxia and isocapnic euoxic recovery of the AHVR test. * significantly lower than baseline ($P \leq 0.05$).

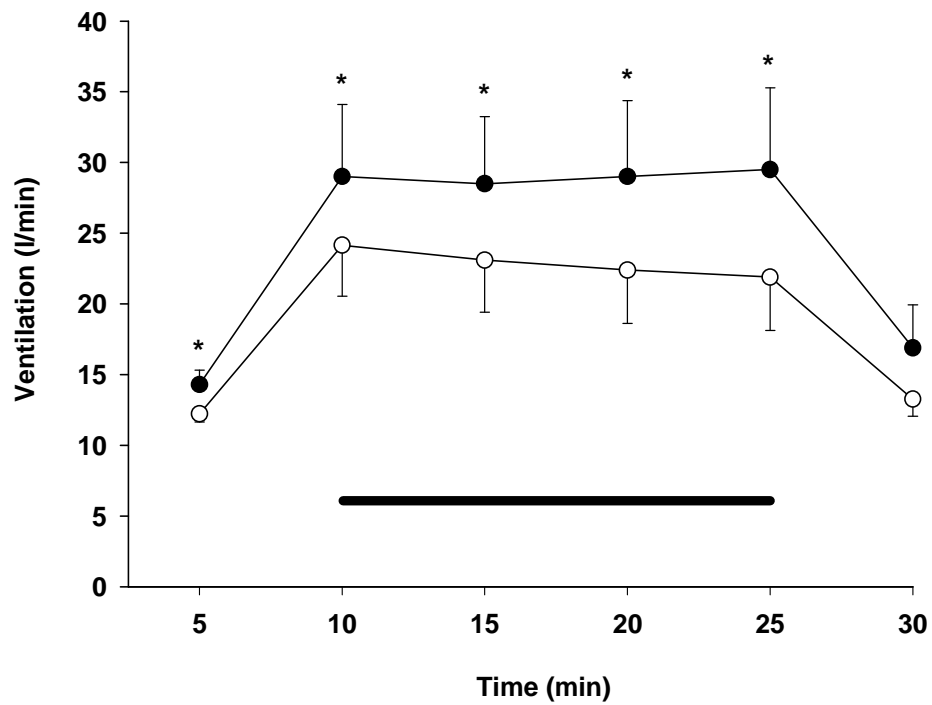


Figure 4.3: Minute ventilation during isocapnic euoxic baseline (5 min), each quarter of isocapnic hypoxia (10,15,20,25 min) and isocapnic euoxic recovery (30 min) of the AHVR test in *Trial 1* (closed symbols) and *Trial 2* (open symbols). * significantly greater than *Trial 2* ($P \leq 0.05$). Thick black line indicates hypoxic exposure.

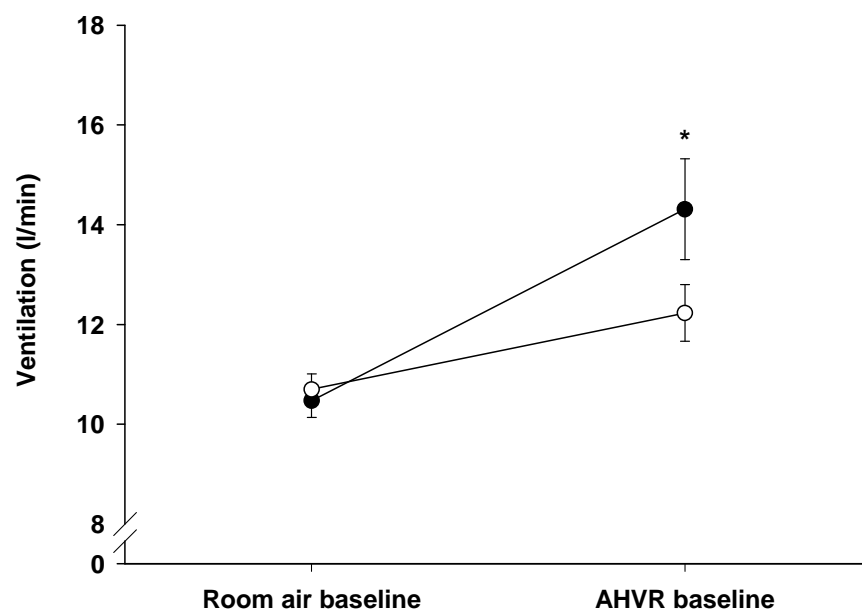


Figure 4.4: Minute ventilation in *Trial 1* (closed symbols) and *Trial 2* (open symbols) during room air baseline and isocapnic euoxic baseline of the AHVR test where P_{ETCO_2} was ~1 mmHg greater than room air baseline. * significantly greater than *Trial 2* ($P \leq 0.05$).

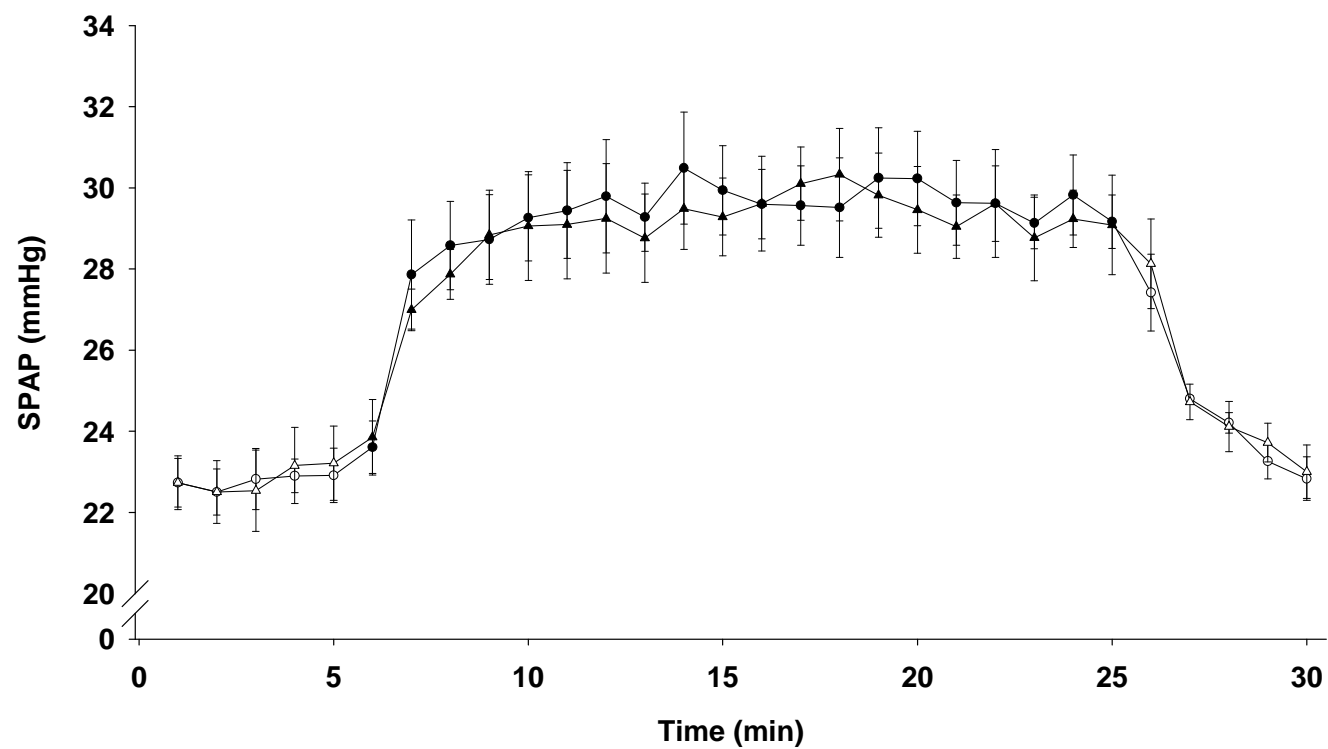


Figure 4.5: Averaged systolic pulmonary arterial pressure (SPAP) during 5 minutes isocapnic euoxic baseline, 20 minutes isocapnic hypoxia and 5 minutes isocapnic euoxic recovery during the AHVR test in *Trial 1* (circles) and *Trial 2* (triangles). Filled symbols indicate recordings were taken during hypoxia.

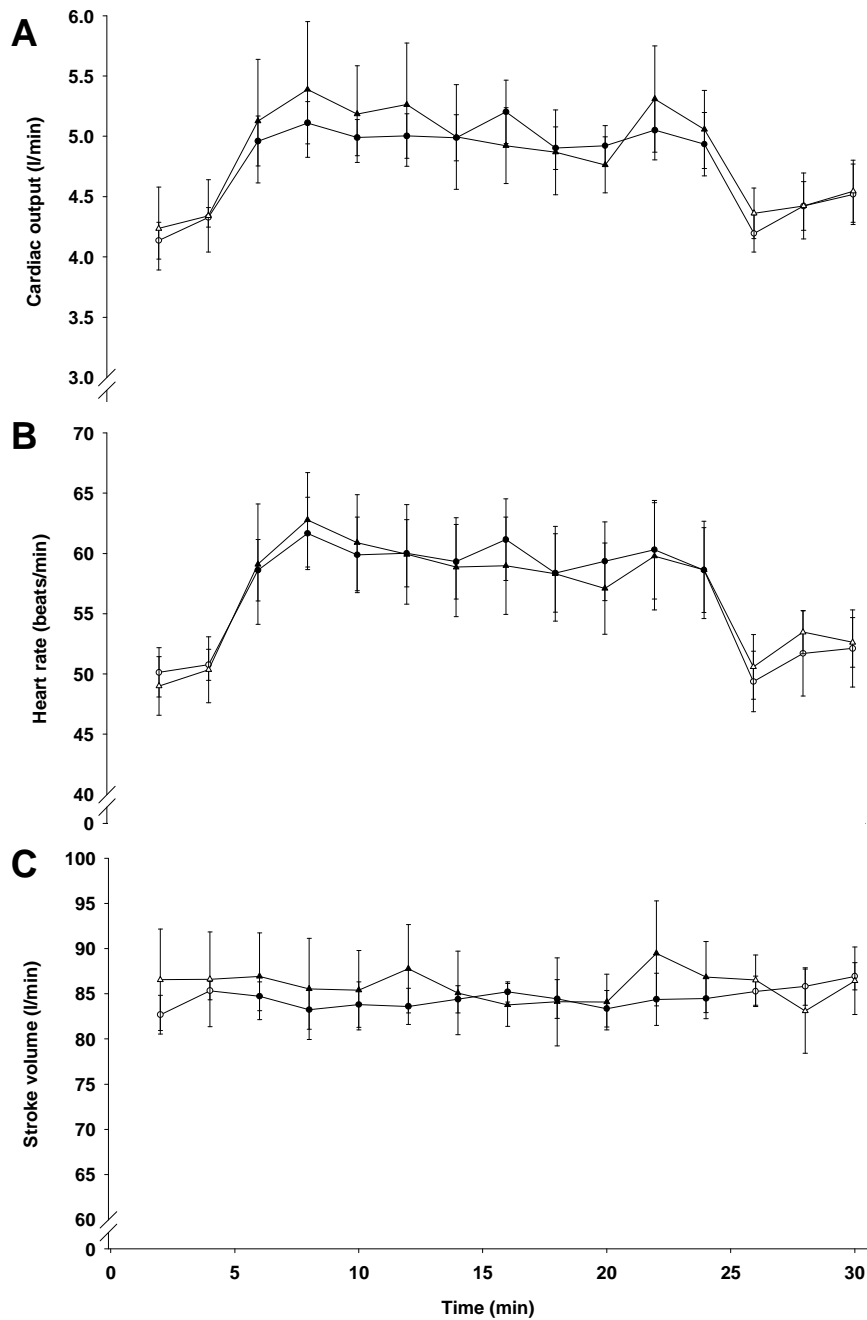


Figure 4.6: Averaged cardiac output (**A**), heart rate (**B**) and stroke volume (**SV**) during 5 minutes isocapnic euoxic baseline, 20 minutes isocapnic hypoxia and 5 minutes isocapnic euoxic recovery in *Trial 1* (circles) and *Trial 2* (triangles). Filled symbols indicate the measurement was taken during hypoxia.

4.4 Discussion

The primary findings of this study are threefold: 1) following exposure to one hour of hypoxic airway occlusions, ventilatory LTF is expressed when P_{ETCO_2} is elevated by ~1 mmHg above normal levels but not during poikilocapnic conditions. Secondly, under the same mildly elevated P_{ETCO_2} conditions augmentation of the HVR is also demonstrated. Thirdly, exposure to hypoxic airway occlusions for one hour does not augment HPV during a subsequent sustained hypoxic exposure.

Respiratory plasticity

Despite a brief period of hyperventilation immediately following the final hypoxic airway occlusion in *Trial 1*, \dot{V}_E quickly returned to baseline levels (< one minute) and then fell lower than baseline levels for the remaining ten minutes of recovery, presumably in response to the hyperventilation-induced hypocapnia. The absence of ventilatory LTF under these poikilocapnic conditions following hypoxic airway occlusions supports the results from previous studies that were unable to demonstrate ventilatory LTF following repetitive hypoxic breath-holds (Cutler et al., 2004b, Cutler et al., 2004a, Leuenberger et al., 2007, Leuenberger et al., 2005). (Harris et al., 2006) postulated that the absence of ventilatory LTF in these studies and numerous other studies of awake humans using intermittent reductions in P_{IO_2} (Mateika and Sandhu, 2011) may have been a result of P_{aCO_2} levels being below the central and peripheral chemoreflex thresholds. Indeed, they were able to

demonstrate the expression of ventilatory LTF in awake humans following an acute bout of IH when PET_{CO_2} was raised above eucapnic baseline levels by 5 mmHg but not during room air breathing. PET_{CO_2} during recovery from the airway occlusions was significantly lower than baseline in *Trial 1* and thus even if Pa_{CO_2} was above the central and peripheral chemoreflex threshold at baseline it may not have been during recovery.

To assess whether PET_{CO_2} levels affected the expression of ventilatory LTF following hypoxic airway occlusions we aimed to elevate PET_{CO_2} by ~1 mmHg above room air baseline levels during the AHVR test. The increase in ventilation during isocapnic euoxic baseline of the AHVR test compared with that of room air baseline in both trials (i.e. prior to exposure of one hour hypoxic airway occlusions in *Trial 1* or unrestricted room air breathing in *Trial 2*) suggests that raising PET_{CO_2} by ~1 mmHg was sufficient to ensure Pa_{CO_2} was above the chemoreflex threshold. Importantly, although PET_{CO_2} was elevated by a similar degree in both trials, \dot{V}_E was increased significantly more in *Trial 1* than *Trial 2* and thus was significantly greater in *Trial 1* during the isocapnic euoxic baseline of the AHVR test. Although we did not expose participants to this ~1 mmHg increase in PET_{CO_2} at baseline and thus cannot compare with that of recovery in each trial, we take the significant trial-by-time interaction to indicate the existence of ventilatory LTF.

Furthermore, \dot{V}_E during the following 20-minute hypoxic exposure was significantly greater in *Trial 1* than *Trial 2*. The greater \dot{V}_E during the sustained hypoxia is indicative of progressive augmentation of the HVR, which is demonstrated in humans exposed to IH during mild hypercapnia but not during eucapnia (Harris et al., 2006). The only study to previously assess whether hypoxic breath-holds caused augmentation of the HVR in awake humans was unable to show any increase during a five-minute exposure to poikilocapnic hypoxia (Leuenberger et al., 2007). As previously discussed in detail (General introduction: *Is progressive augmentation of the HVR the same phenomenon as respiratory LTF and what is the responsible mechanism(s)*) progressive augmentation of the HVR and ventilatory LTF may simply be the same form of respiratory plasticity just demonstrated under different O_2 tensions but we are unaware how our data can be used to support or refute this hypothesis.

We are aware that a major limitation of our study was the absence of a trial that included airway occlusions without hypoxia, such as was performed in chapter 3. Therefore, it remains a possibility that other aspects of the airway occlusions (e.g. intermittent hypercapnia, lung receptor mechanical feedback and negative intrathoracic pressure) could have mediated ventilatory LTF and/or progressive augmentation of the HVR independently of IH. It would seem unlikely that hypercapnia occurring during each airway occlusion could have mediated the respiratory plasticity as previous animal evidence demonstrates it is not a stimulus

capable of inducing respiratory plasticity (Baker et al., 2001). It should be noted however, that we are unaware of any study that has examined whether exposure to intermittent hypercapnia evokes respiratory LTF in humans. Furthermore, it would seem unlikely that respiratory plasticity is the result of enhanced inspiratory activation (i.e. post airway occlusion hyperventilation). Postnatal rats demonstrate substantial hypoxic ventilatory decline and thus during hypoxic exposures they do not experience a notable increase in ventilation but still develop a significant magnitude of respiratory LTF (Tadjalli et al., 2007). During lung inflation sensory information from lung mechanoreceptors are relayed to the respiratory control centre in the brainstem via the vagus nerve (Ezure et al., 2002, Vanlunteren et al., 1984, Kubin et al., 2006). Interestingly, (Tadjalli et al., 2010) recently demonstrated in rats that a reduction in vagal feedback activity during airway occlusions evoked LTF of hypoglossal nerve activity but had no sustained effect on diaphragm activity. Therefore, it would seem unlikely that intermittent reduction in vagal activity from lung mechanoreceptors during each airway occlusion in our study would have mediated the ventilatory LTF. However, this remains to be investigated in humans and thus remains a possibility. Furthermore, (Ryan and Nolan, 2009b) exposed anaesthetised rats to repetitive, five second episodes of negative upper airway pressure without concurrent hypoxia and was able to demonstrate the manifestation of LTF of diaphragm activity. Therefore, it is possible that the negative intrathoracic pressures generated during each occluded inspiratory effort may have mediated the ventilatory LTF independent of hypoxia in our study. Neither (Tadjalli et al., 2010) nor (Ryan and Nolan, 2009b) examined whether

exposure to airway occlusions or intermittent negative upper airway pressures caused progressive augmentation of the HVR. Therefore, it is not known whether either an intermittent reduction in vagal activity or negative upper airway pressures could have mediated the progressive augmentation shown in our study.

Hypoxic pulmonary vasoconstriction

It has previously been shown in humans that prior exposure to eight hours of sustained hypoxia sensitises the HPV response to a subsequent brief (25 minutes) hypoxic exposure (Smith et al., 2008a). HPV is known to be in part regulated by hypoxia-inducible factor (HIF) that controls the expression of a multitude of proteins, some of which may affect the response of the pulmonary vasculature to hypoxia (Smith et al., 2008b). It would appear that HIF plays a primary role in mediating the increased HPV sensitivity following prior exposure as iron supplementation abolished the augmentation in HPV and furthermore, iron depletion with infusion of desferrioxamine in the absence of hypoxia was able to mimic the effect of eight hours exposure to sustained hypoxia (Smith et al., 2008a). It remains unknown whether exposure to IH as occurs in OSA is capable of activating the HIF pathway and sensitising HPV to a subsequent hypoxic exposure as no human study has been performed to examine HPV during or after IH. Interestingly, in anaesthetised dogs HPV has been shown to gradually increase during successive intermittent exposures to 15 minutes of hypoxia, each interspersed by 15 minutes euoxia (Unger et al., 1977). Hypoxic exposures during airway occlusions in our study were brief compared to the 15 minute episodes

used in Unger's et al., (1977) study and the total duration of the hypoxic exposures was far shorter than the eight hours used by (Smith et al., 2008a). However, we thought it was still possible that HPV during a 20-minute hypoxic exposure would be greater following one hour of hypoxic airway occlusions than following one hour of unrestrained room air breathing.

Having only performed measurements of SPAP in five of the eleven participants in this study we must be conservative with our conclusions. However, in none of the five participants was there an indication for any augmentation in SPAP during the AHVR test following hypoxic airway occlusions. In humans, HPV consists of two distinct phases, a relatively rapid phase that reaches a plateau within several minutes and a second more gradual phase that is not initiated for ~40 minutes (Talbot et al., 2005) and does not reach a plateau for several hours (Dorrington et al., 1997). The initial rapid phase of HPV is clearly shown in our data and the plateau is similar between the two trials. Furthermore, in both trials this steady state plateau remains until the end of the 20 minute hypoxic exposure. Therefore, it is clear that the prior exposure to hypoxic airway occlusions did not cause any sensitisation of HPV. It is likely that the duration of airway occlusions in our study was too brief and that a longer exposure similar to what OSA patients experience nightly could have caused a notable augmentation of HPV.

Summary

In summary, ventilatory LTF was not evident during ten minutes of room air recovery following one hour of hypoxic airway occlusions but did manifest when P_{ETCO_2} levels were increased above baseline levels by ~1 mmHg. Furthermore, \dot{V}_E during the 20 minute hypoxic exposure following one hour of hypoxic airway occlusions was enhanced compared with prior room air breathing suggesting the expression of progressive augmentation of the HVR. Future studies are required to determine whether hypoxia *per se* or negative pressures and/or reduction in lung mechanoreceptor feedback during airway occlusions mediates this respiratory plasticity. HPV during a 20 minute exposure to sustained hypoxia was unaltered by prior exposure to one hour of hypoxic airway occlusions. Future studies are required to see if longer exposures to OSA-related IH are capable of sensitising the HPV response as this may have implications for OSA patients with COPD.

5 CHAPTER 5 - LONG-TERM FACILITATION OF VENTILATION FOLLOWING ACUTE CONTINUOUS HYPOXIA IN AWAKE HUMANS DURING SUSTAINED HYPERCAPNIA

5.1 Introduction

A great number of studies have been undertaken during the last three decades which have contributed to our understanding of the mechanisms that may be involved in initiating and maintaining IH-induced respiratory LTF in animals. In contrast, despite a number of studies demonstrating the development of respiratory LTF in humans following intermittent reductions in P_{iO_2} (Harris et al., 2006, Wadhwa et al., 2008, Lee et al., 2009, Gerst et al., 2011) and following hypoxic airway occlusions (chapter 4) there has been almost no research conducted to investigate the responsible mechanisms in humans.

The carotid body (CB) is the primary O_2 sensor in mammals and increased afferent discharge during hypoxia is considered as a logical initiating step in the development of respiratory LTF. Indeed, episodic stimulation of the cut carotid sinus nerve of rodents can initiate respiratory LTF (Millhorn et al., 1980a, Hayashi et al., 1993). However, respiratory LTF is sustained long after cessation of carotid sinus nerve stimulation suggesting that it is a central mechanism(s) that maintains respiratory LTF rather than CB afferent discharge (Hayashi et al., 1993, Millhorn et

al., 1980a). Furthermore, direct recordings of rodent (*in* and *ex vivo*) CB discharge show a return to baseline levels immediately upon each re-oxygenation following acute IH (Peng et al., 2003) and respiratory LTF is evident in rodents following acute IH despite CB inhibition with hyperoxia (Bach and Mitchell, 1996, Xing and Pilowsky, 2010, Baker and Mitchell, 2000). Thus, although strong evidence exists to suggest CB afferent discharge plays a primary role in initiating respiratory LTF during acute IH in rodents, it is generally accepted that it does not have an active role in maintaining respiratory LTF beyond hypoxic cessation.

In humans, elevated sympathetic nervous activity during acute IH or acute CH is sustained for up to three hours following resumption of normal inspired O₂ levels (Morgan et al., 1995, Xie et al., 2001, Xie et al., 2000, Tamisier et al., 2005, Cutler et al., 2004b, Cutler et al., 2004a, Querido et al., 2010, Leuenberger et al., 2005). It has recently been demonstrated that this sympathetic LTF following acute CH is transiently attenuated during brief inhibition of CB afferent discharge with hyperoxia (Querido et al., 2010). Because the pattern of sympathetic activity in humans (Seals et al., 1993) and rodents (Dick et al., 2004) is tightly coupled to that of the central respiratory drive we hypothesised that in awake humans, the CB discharge necessary for maintaining the full expression of sympathetic LTF may also maintain respiratory LTF following acute CH and acute IH. Currently, there is no available data to specifically link CB activity in the maintenance of ventilatory LTF in humans and this remains to be determined. The absence of ventilatory LTF following acute CH may simply be because acute CH is a stimulus that does not

elicit respiratory LTF in awake humans. This would be in agreement with previous animal research that has failed to link acute CH with respiratory LTF (Baker and Mitchell, 2000, Mitchell et al., 2001b, Turner and Mitchell, 1997, Dwinell et al., 1997, McKay et al., 2004). Alternatively, ventilatory LTF following acute CH may not have been evident in previous human studies (Querido et al., 2010, Tamisier et al., 2005, McEvoy et al., 1996, Morgan et al., 1995, Xie et al., 2001) because PaCO_2 levels were not maintained above eucapnic levels. Indeed, ventilatory LTF is not expressed in humans following acute IH (McEvoy et al., 1996, Jordan et al., 2002, Mateika et al., 2004, Khodadadeh et al., 2006, Diep et al., 2007) unless CO_2 levels are elevated and maintained above eucapnic levels (Harris et al., 2006, Wadhwa et al., 2008, Lee et al., 2009, Gerst et al., 2011). Furthermore, we have previously shown in chapter 4 that ventilatory LTF is only expressed in awake humans following one hour of hypoxic airway occlusions when PETCO_2 was elevated above normal levels.

Study aims

We aimed to examine whether acute CH of the same total duration of hypoxia as acute IH was able to elicit ventilatory LTF when CO_2 levels were maintained above eucapnic levels. In addition, by transiently inhibiting CB discharge with brief hyperoxic exposures we aimed to investigate whether CB discharge was involved in the maintenance of ventilatory LTF following acute IH and acute CH.

5.2 Methods

Ethical approval

After receiving detailed information on the procedures and risks all participants gave written consent to take part. The study was performed according to the latest revision of the Declaration of Helsinki and was approved by the local ethics committee (University of Birmingham ethical review committee).

Participants

Twelve healthy male subjects participated in the study. All subjects were non-smokers, had no history of cardiovascular, respiratory and metabolic disease and they were not taking any medication.

Protocol

Participants visited the laboratory for three experimental trials more than 24 hours apart; *Trial 1* - acute IH, *Trial 2* - acute CH, and *Trial 3* - Control. Prior to experimental visits participants also undertook a preliminary visit to familiarize themselves with the instrumentation and exposure to the various gas mixtures. For all experimental trials participants were asked to refrain from alcohol consumption and moderate to vigorous exercise for 24 hours and caffeine intake for 12 hours prior to arriving at the laboratory. In addition all experimental trials were performed

at least three hours after food consumption. All trials were performed at the same time of day and were performed in a randomised order.

Preliminary visit

On arrival at the laboratory participants positioned themselves comfortably on a couch in the supine position where they were exposed to two 4-minute episodes of hypoxia during elevations in CO₂ as would be experienced in *Trial 1* and described in the next section. Instrumentation for this visit was identical to all other experimental visits as described below (Instrumentation).

Trial 1: Once positioned comfortably on the couch participants breathed room air (RA) for 15 minutes in order to make eucapnic baseline measurements, followed by one minute exposure to hyperoxia. Subsequently, PETCO₂ was elevated by 4-5 mmHg above eucapnic levels and it was maintained at this level until the start of eucapnic recovery as described below. During the elevation in PETCO₂, PETO₂ was maintained at a euoxic level (100 mmHg), except for during hypoxic and hyperoxic exposures. After 20 minutes of acclimatisation to the newly elevated PETCO₂ (hypercapnic baseline), participants were exposed to one minute of hyperoxia followed by an additional two minutes recovery to restore euoxia. Subsequently, participants experienced eight, 4-minute episodes of hypoxia each separated by 4-minute periods of euoxia. Following the final hypoxic episode, hypercapnic recovery consisted of four 5-minute periods, (R1-R4), each followed by one minute

of hyperoxia. After an additional two-minute recovery to restore euoxia following the final hyperoxia, inspired CO₂ was removed to enable 15 minutes of eucapnic recovery, followed by a final one-minute exposure to hyperoxia. See Figure 5.1 for an illustration of the protocol.

Trial 2: *Trial 2* was identical to that of *Trial 1*, except the eight, 4-minute hypoxic episodes were substituted with one continuous hypoxic episode of the same total duration (32 minutes). See Figure 5.1 for an illustration of the protocol.

Trial 3: In *Trial 3* euoxia was maintained throughout the entire protocol and was otherwise identical to *Trial 1*, thus serving as a control for the sustained elevation in PETCO₂ experienced in *Trial 1* and *Trial 2*. See Figure 5.1 for an illustration of the protocol.

Instrumentation

Participants wore a comfortable face mask that allowed breathing through either the mouth or the nose (Hans Rudolph 7450 Series V2 oro-nasal mask, total dead space 100-125 ml depending on facemask size). The facemask was connected to the gas supply with a short flexible tube that allowed small head movements without lifting of the facemask seal. A good seal was ensured at all times by careful observation of gas waveforms on the computer monitor. End-tidal gas was sampled continuously from a catheter within the facemask. Ventilation was

measured and PET_{O_2} and PET_{CO_2} were controlled in an identical fashion to that previously described in chapter 3.

During hypoxia in *Trial 1* and *Trial 3*, PET_{O_2} was rapidly reduced to 50 mmHg. Upon completion of each of the eight hypoxic episodes in *Trial 1* and the 32 minutes of continuous hypoxia in *Trial 2*, PET_{O_2} was rapidly returned to 100 mmHg. The gas inspired during the exposures to hyperoxia consisted of 100% O_2 with additional CO_2 to maintain PET_{CO_2} at the desired hypercapnic level. Hyperoxic exposures during eucapnic baseline and eucapnic recovery involved 100% inspired O_2 without any additional CO_2 .

Throughout all trials arterial O_2 saturation (S_aO_2) was continuously measured using a pulse oximeter, worn on the participant's ear lobe (Datex-Ohmeda 3900).

Ventilatory analysis

In all experimental trials, \dot{V}_E , S_aO_2 , PET_{CO_2} and PET_{O_2} were recorded continuously. In all trials, baseline ventilatory measurements were made during both the eucapnic and hypercapnic periods by averaging \dot{V}_E for the last three minutes of each period. Likewise, in all trials, ventilatory measurements were made every five minutes during both the hypercapnic and eucapnic recovery periods by averaging \dot{V}_E for the last three minutes of each five-minute segment. Ventilatory measurements during the intervention period in *Trial 1* (acute IH) were made for

each hypoxic and euoxic interval by averaging \dot{V}_E for the last two minutes of each interval. In *Trial 2* and *Trial 3* ventilatory measurements during the intervention periods (ACH and euoxia, respectively) were made every four minutes to match the measurement points in *Trial 1*.

To assess the effect of inhibition of CB afferent discharge, ventilatory measurements were averaged over the entire minute of each hyperoxic exposure and compared with the ventilatory measurements made during the immediately preceding period of euoxia. The initial two breaths of 100% inspired O_2 were excluded from the analysis to account for lung-to-CB circulation delay.

Statistical analysis

A two-way analysis of variance (ANOVA) with repeated measures in conjunction with *post hoc* LSD test was used to assess whether ventilatory measurements (\dot{V}_E , PET_{CO_2} , PET_{O_2} and S_aO_2) during eucapnic and hypercapnic recovery were significantly different from their respective baselines within each trial and whether there were significant differences between trials. In addition, the same statistical approach was used to assess whether the hyperoxia-induced reduction in \dot{V}_E during hypercapnic recovery was significantly different from that during hypercapnic baseline within and between each trial. Finally, in *Trial 1* a one-way ANOVA with repeated measures was used to assess whether there were significant differences between ventilatory measurements during the eight hypoxic

episodes and also during the eight immediately preceding euoxic episodes. Values are expressed as mean \pm standard error and differences were considered significant if $P \leq 0.05$.

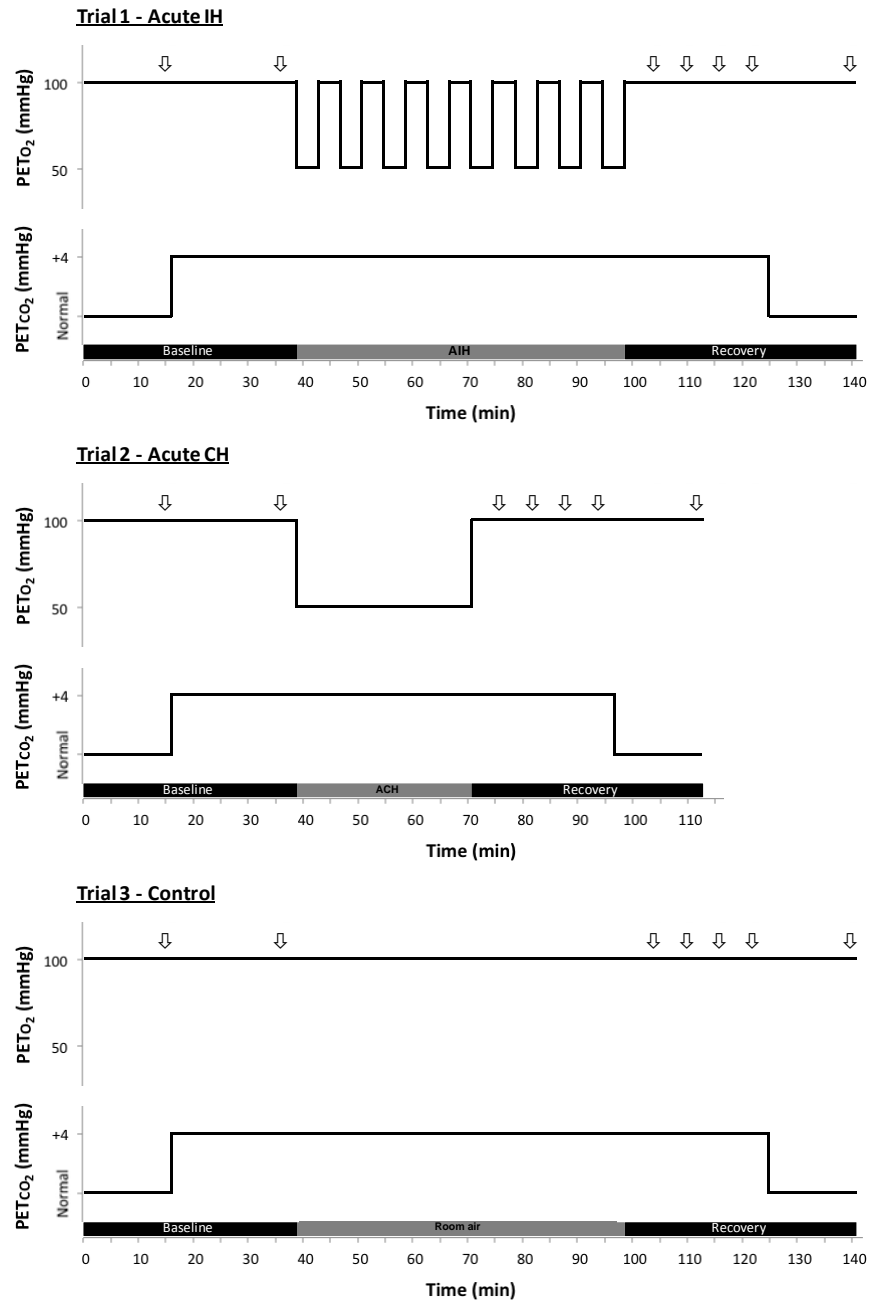


Figure 5.1: Schematic demonstrating protocols performed in *Trial 1*, *2* and *3*. Arrows denote one-minute exposures to 100% inspired O_2 .

5.3 Results

Participants

Twelve healthy male participants completed the experiment with an average age of 23.5 ± 0.42 years, weight of 77.3 ± 2.4 Kg, height of 180.8 ± 1.8 cm and BMI of 23.6 ± 0.56 . All values are mean \pm standard deviation.

Gas control

Figure 5.2 shows the PET_{O_2} and PET_{CO_2} that were achieved during all trials. The elevation in PET_{CO_2} above eucapnic levels during the periods of hypercapnia in all trials was near identical (*Trial 1*: 4.6 ± 0.2 , *Trial 2*: 4.6 ± 0.2 , *Trial 3*: 4.4 ± 0.2 mmHg). PET_{CO_2} was controlled with considerable precision during all hypercapnic periods including when ventilation was altered due to superimposed hypoxia or hyperoxia. PET_{O_2} was maintained at 100 mmHg during all periods of euoxia and at 50 mmHg during all periods of hypoxia. Inspiration of 100% O_2 was successful in elevating PET_{O_2} well above the threshold that is required to inhibit CB afferent discharge. Tables 5.1-4 list the average end-tidal partial pressures achieved in all trials.

Ventilation

Euoxia: In *Trial 1*, \dot{V}_E was significantly elevated above hypercapnic baseline during the entire hypercapnic recovery period (Figure 5.3). Intriguingly, in *Trial 2*, \dot{V}_E during the entire hypercapnic recovery period was also significantly elevated above baseline of an equivalent magnitude to that of *Trial 1* (Figure 5.4). Furthermore, in both trials, \dot{V}_E continued to increase during hypercapnic recovery and was significantly greater during the final quarter than in the initial quarter (Figure 5.3 and 5.4).

However, in *Trial 3* where participants were exposed to hypercapnia without hypoxia, \dot{V}_E during the hypercapnic recovery period was also significantly greater than hypercapnic baseline (Figure 5.5). Unlike *Trial 1* and *Trial 2*, \dot{V}_E did not increase further during the hypercapnic recovery period but remained constant in *Trial 3* (Figure 5.5). The magnitude of the increase in \dot{V}_E from baseline to the final quarter of the recovery period was significantly greater in *Trial 1* and *Trial 2* (Figure 5.6).

In *trial 1*, \dot{V}_E during the later hypoxic episodes began to increase and the final hypoxic episode was significantly greater than that of the first (Figure 5.3). \dot{V}_E during each euoxic episode that immediately preceded the eight hypoxic episodes did not change over time.

In all trials \dot{V}_E during eucapnic recovery had returned to eucapnic baseline levels within the first or second minute and remained at this level throughout the entire 15-minute period (Figures 5.3-5.5).

Hyperoxia: In all trials each exposure to hyperoxia significantly reduced \dot{V}_E (Figures 5.3-5.5). The hyperoxia-induced reduction in \dot{V}_E was greater during concurrent hypercapnia, when CB activity would be expected to be elevated above that of room air breathing (Figures 5.3-5.5).

In contrast to our hypothesis that CB afferent discharge would be actively involved in maintaining ventilatory LTF, inhibition of the CB with hyperoxia did not abolish or even attenuate the apparent ventilatory LTF. Hyperoxia reduced ventilation to the same extent before and following the intervention in each trial (e.g. acute IH, acute CH or Control) (Figure 5.7) meaning that the increase in \dot{V}_E observed in all trials in the period following acute IH, acute CH or Control was independent of CB discharge (Figure 5.6).

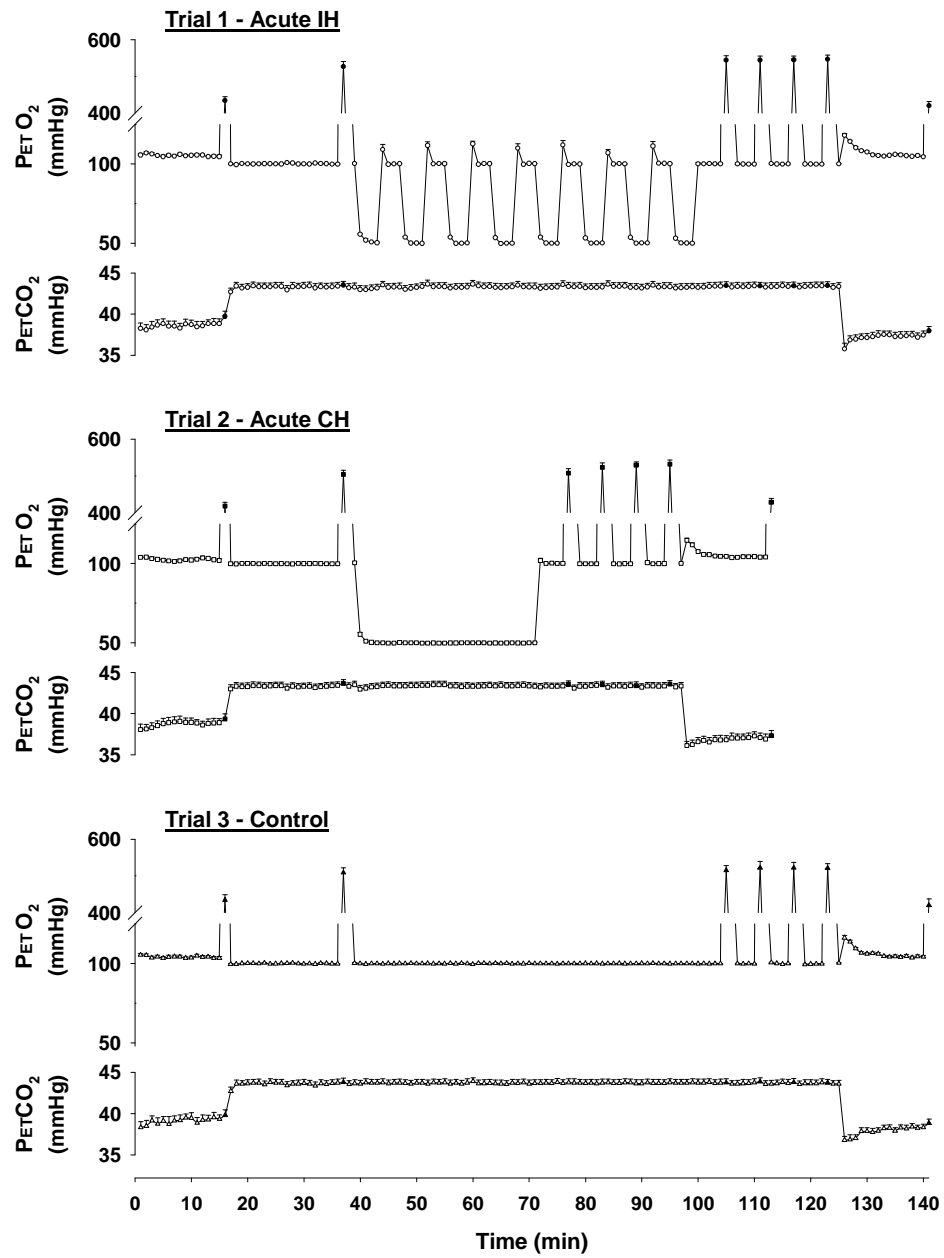


Figure 5.2: Minute averages for PETO₂ and PETCO₂ for each trial. Filled symbols indicate hyperoxic exposures.

Trial 1 - (acute IH)				
Time	EB	ER 5 min	ER 10 min	ER 15 min
PETCO ₂ (mmHg)	38.8 ± 0.6	37.1 ± 0.5 *	37.4 ± 0.5 *	37.4 ± 0.5 *
PETO ₂ (mmHg)	104 ± 0.8	109 ± 0.7 *	105 ± 0.6	105 ± 0.6
S _a O ₂ (%)	97.5 ± 0.2	97.5 ± 0.2	97.3 ± 0.2	97.3 ± 0.2
Trial 2 - (acute CH)				
Time	EB	ER 5 min	ER 10 min	ER 15 min
PETCO ₂ (mmHg)	38.9 ± 0.5	36.6 ± 0.5 *	37.0 ± 0.5 *	37.1 ± 0.6 *
PETO ₂ (mmHg)	103 ± 0.9	106 ± 0.7 *	104 ± 0.6	104 ± 0.8
S _a O ₂ (%)	97.3 ± 0.2	97.4 ± 0.1	97.1 ± 0.8	97.3 ± 0.2
Trial 3 - (control)				
Time	EB	ER 5 min	ER 10 min	ER 15 min
PETCO ₂ (mmHg)	39.4 ± 0.5	37.6 ± 0.4 *	38.1 ± 0.4 *	38.3 ± 0.4 *
PETO ₂ (mmHg)	103 ± 1.0	107 ± 0.7 *	104 ± 0.8	104 ± 0.8
S _a O ₂ (%)	97.8 ± 0.2	97.6 ± 0.2	97.4 ± 0.2	97.3 ± 0.2

Table 5.1: Averaged PETCO₂, PETO₂ and S_aO₂ values during eucapnic baseline (EB) and eucapnic recovery (ER). * significantly different from eucapnic baseline.

Trial 1 - (acute IH)		
Time	EB	ER
PETCO ₂ (mmHg)	39.7 ± 0.6	38.0 ± 0.5 *
PETO ₂ (mmHg)	433 ± 10	420 ± 11.9
S _a O ₂ (%)	99.0 ± 0.2	98.8 ± 0.2
Trial 2 - (acute CH)		
Time	EB	ER
PETCO ₂ (mmHg)	39.4 ± 0.6	37.3 ± 0.6 *
PETO ₂ (mmHg)	418 ± 11	428 ± 10
S _a O ₂ (%)	98.8 ± 0.2	98.9 ± 0.2
Trial 3 - (control)		
Time	EB	ER
PETCO ₂ (mmHg)	39.8 ± 0.7	38.4 ± 0.5 *
PETO ₂ (mmHg)	434 ± 14	420 ± 18
S _a O ₂ (%)	99.1 ± 0.1	98.9 ± 0.2

Table 5.2: Averaged PETCO₂, PETO₂ and S_aO₂ values for hyperoxic exposures during eucapnic baseline (EB) and eucapnic recovery (ER). * significantly lower than EB.

Trial 1 - (acute IH)					
Time	HB	HR 5 min	HR 10 min	HR 15 min	HR 20 min
PETCO ₂ (mmHg)	43.4 ± 0.5	43.4 ± 0.5	43.4 ± 0.5	43.4 ± 0.5	43.4 ± 0.5
PETO ₂ (mmHg)	100 ± 0.1	100 ± 0.18	100 ± 0.04	100 ± 0.1	100 ± 0.05
S _a O ₂ (%)	97.5 ± 0.1	97.5 ± 0.2	97.3 ± 0.2	97.2 ± 0.2	97.3 ± 0.2
Trial 2 - (acute CH)					
Time	HB	HR 5 min	HR 10 min	HR 15 min	HR 20 min
PETCO ₂ (mmHg)	43.4 ± 0.4	43.3 ± 0.4	43.4 ± 0.4	43.4 ± 0.4	43.3 ± 0.4
PETO ₂ (mmHg)	100 ± 0.1	100 ± 0.1	100 ± 0.04	100 ± 0.05	100 ± 0.03
S _a O ₂ (%)	97.3 ± 0.2	97.5 ± 0.2	97.6 ± 0.2	97.5 ± 0.2	94.5 ± 0.2
Trial 3 - (control)					
Time	HB	HR 5 min	HR 10 min	HR 15 min	HR 20 min
PETCO ₂ (mmHg)	43.7 ± 0.4	43.7 ± 0.4	43.7 ± 0.5	43.7 ± 0.4	43.7 ± 0.4
PETO ₂ (mmHg)	100 ± 0.1	100 ± 0.05	100 ± 0.1	100 ± 0.1	100 ± 0.1
S _a O ₂ (%)	97.3 ± 0.1	97.6 ± 0.1	97.4 ± 0.1	97.6 ± 0.2	97.6 ± 0.2

Table 5.3: Averaged PETCO₂, PETO₂ and S_aO₂ values during hypercapnic baseline (HB) and hypercapnic recovery (HR).

Trial 1 - (acute IH)					
Time	HB	HR 5 min	HR 10 min	HR 15 min	HR 20 min
PETCO ₂ (mmHg)	43.5 ± 0.5	43.5 ± 0.5	43.4 ± 0.5	43.4 ± 0.5	43.5 ± 0.5
PETO ₂ (mmHg)	526 ± 14	544 ± 10	544 ± 11	545 ± 12	547 ± 11
S _a O ₂ (%)	99.2 ± 0.2	98.9 ± 0.1	98.9 ± 0.1	98.8 ± 0.2	98.7 ± 0.1
Trial 2 - (acute CH)					
Time	HB	HR 5 min	HR 10 min	HR 15 min	HR 20 min
PETCO ₂ (mmHg)	43.6 ± 0.5	43.6 ± 0.4	43.5 ± 0.4	43.4 ± 0.4	43.6 ± 0.4
PETO ₂ (mmHg)	504 ± 11	507 ± 12	524 ± 12	529 ± 9	532 ± 11
S _a O ₂ (%)	98.8 ± 0.2	98.9 ± 0.2	99.1 ± 0.1	99.0 ± 0.2	99.0 ± 0.2
Trial 3 - (control)					
Time	HB	HR 5 min	HR 10 min	HR 15 min	HR 20 min
PETCO ₂ (mmHg)	43.8 ± 0.5	43.8 ± 0.5	43.9 ± 0.5	43.8 ± 0.4	43.7 ± 0.4
PETO ₂ (mmHg)	509 ± 13	515 ± 15	522 ± 17	521 ± 16	521 ± 12
S _a O ₂ (%)	99.1 ± 0.2	98.9 ± 0.2	99.1 ± 0.1	99.1 ± 0.2	99.2 ± 0.2

Table 5.4: Averaged PETCO₂, PETO₂ and S_aO₂ values for minute hyperoxic exposures during hypercapnic baseline (HB) and hypercapnic recovery (HR).

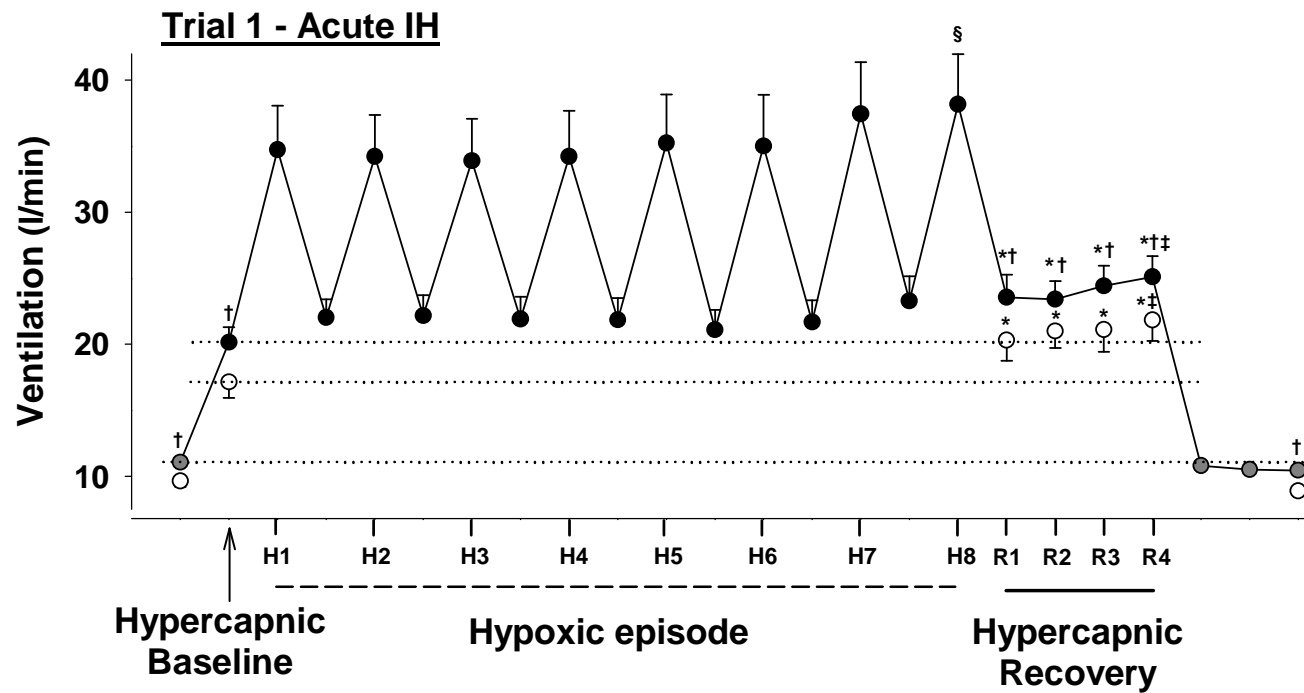


Figure 5.3: (Trial 1) Black circles indicate average values for minute ventilation during hypercapnic baseline, each hypoxic and euoxic interval during IH and hypercapnic recovery. Grey circles indicate average values for ventilation during eucapnic baseline and during eucapnic recovery. White circles indicate average ventilation during hyperoxic exposures which immediately followed that of the euoxic time point positioned above (as indicated by a black circle). * significantly greater than respective baseline (i.e. euoxic or hyperoxic). † significantly greater than during the corresponding hyperoxic exposure. ‡ significantly greater than the start of hypercapnic recovery. § significantly greater than the first hypoxic episode.

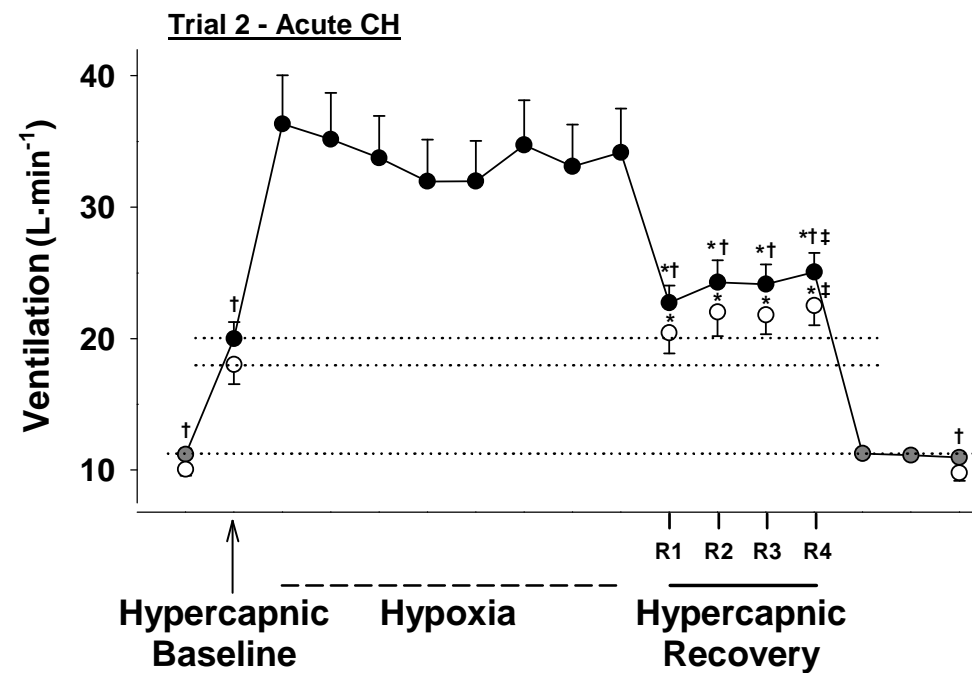


Figure 5.4: (Trial 2) Black circles indicate average values for minute ventilation during hypercapnic baseline, continuous hypoxia and hypercapnic recovery. Grey circles indicate average values for ventilation during eucapnic baseline and during eucapnic recovery. White circles indicate average ventilation during hyperoxic exposures which immediately followed that of the euoxic time point positioned above (as indicated by a black circle). * significantly greater than respective baseline (i.e. euoxic or hyperoxic). † significantly greater than during the corresponding hyperoxic exposure. ‡ significantly greater than the start of hypercapnic recovery.

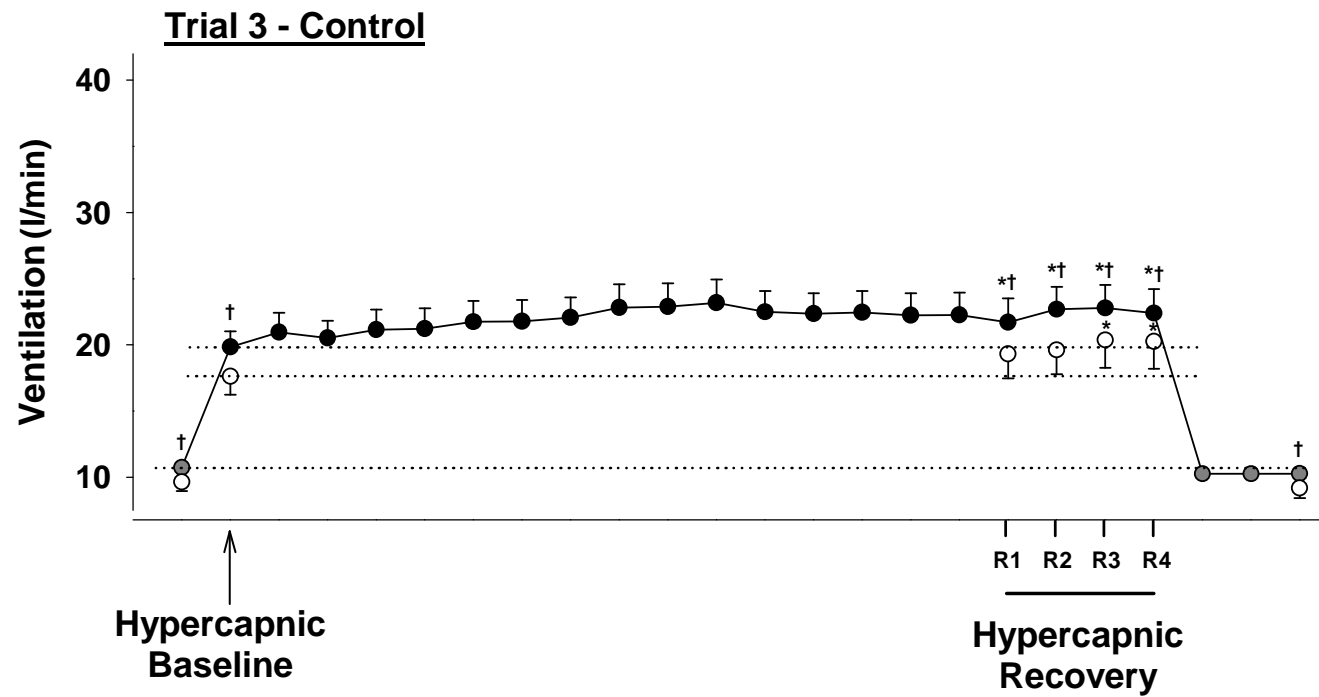


Figure 5.5: (Trial 3) Black circles indicate average values for minute ventilation during hypercapnic baseline, continuous euoxia and hypercapnic recovery. Grey circles indicate average values for ventilation during eucapnic baseline and during eucapnic recovery. White circles indicate average ventilation during hyperoxic exposures which immediately followed that of the euoxic time point positioned above (as indicated by a black circle). * significantly greater than respective baseline (i.e. euoxic or hyperoxic). † significantly greater than during the corresponding hyperoxic exposure.

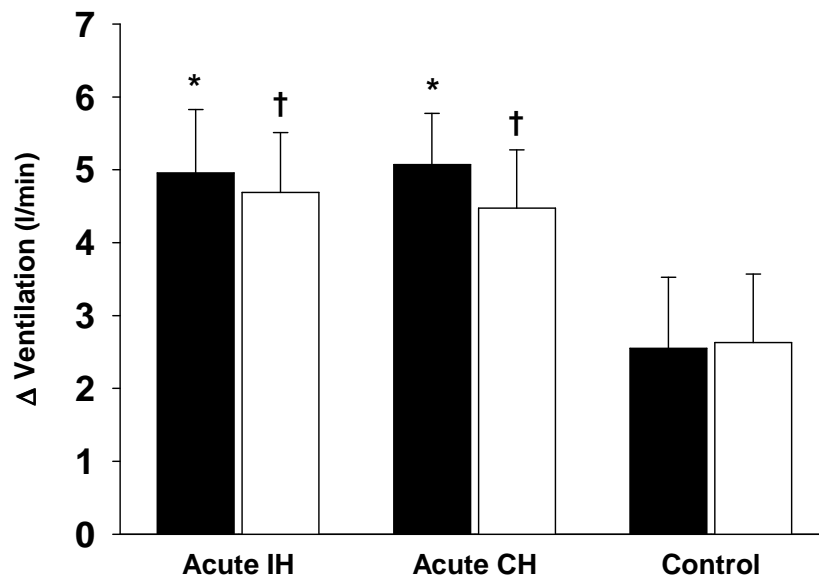


Figure 5.6: Change in minute ventilation from hypercapnic baseline to the final quarter of recovery, during euoxia (black columns) and hyperoxia (white columns). * significantly greater than control trial during euoxia, † significantly greater than control trial during hyperoxia.

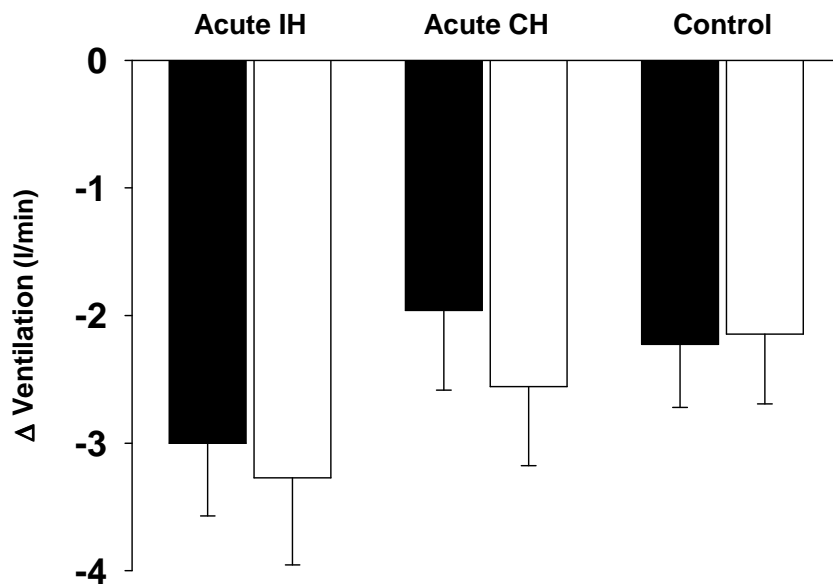


Figure 5.7: Hyperoxia-induced reduction in minute ventilation during hypercapnic baseline (black columns) and during the final quarter of hypercapnic recovery (white columns).

5.4 Discussion

The primary findings of this study are three fold: 1) in awake humans, exposure to acute CH during hypercapnia induces a similar sustained elevation in ventilation to that of exposure to acute IH, 2) approximately half of this elevation in ventilation may be attributed to ventilatory LTF as exposure to sustained hypercapnia causes a gradual increase in ventilation that is independent of hypoxic exposure, 3) hyperoxia reduced ventilation to the same extent before and following acute IH and acute CH suggesting the CB is not actively involved in maintaining ventilatory LTF in awake humans.

Hypercapnia and respiratory LTF

Although respiratory LTF has been repeatedly demonstrated in a range of animal species using various preparations since the first publication in 1980 (Millhorn et al., 1980a), evidence for its existence in awake humans remained elusive until relatively recently despite the completion of numerous studies designed to investigate this phenomenon (Mateika and Sandhu, 2011). As discussed previously, (chapter 1 and 4) ventilatory LTF may be restrained in these studies of awake humans because P_{aCO_2} may have been below the chemoreflex threshold. By raising P_{ETCO_2} 5 mmHg above eucapnic levels and maintaining this throughout acute IH and recovery, (Harris et al., 2006) was able to demonstrate the manifestation of LTF in awake humans. Furthermore, in chapter 4 we were able to

demonstrate ventilatory LTF in awake humans following one hour of hypoxic airway occlusions during room air breathing when P_{ETCO_2} was elevated ~ 1 mmHg above normal levels but not during room air breathing.

Using an almost identical acute IH protocol and a similar level of elevated P_{ETCO_2} as used by (Harris et al., 2006) we demonstrated the appearance of ventilatory LTF of a similar magnitude in a population of young males. Furthermore, to the best of our knowledge we are the first to investigate whether acute CH also evokes ventilatory LTF during elevated P_{ETCO_2} levels in awake humans. In contrast to a substantial body of evidence in animal research showing that only acute IH produces respiratory LTF we demonstrated an equivalent magnitude of ventilatory LTF with acute CH to that following acute IH. However, our results from *Trial 3* (Control) also demonstrated a gradual but significant rise in ventilation during sustained elevations in P_{ETCO_2} that was independent of the exposure to hypoxia. Although the increase in \dot{V}_E was considerably less, at approximately half of the increase demonstrated in *Trial 1* and *Trial 2* following acute IH and acute CH, we believe that the increase in ventilation due to sustained hypercapnia must account for part of the apparent ventilatory LTF following both acute IH and acute CH.

The possibility that the apparent ventilatory LTF following acute IH is not exclusively dependent on the exposure to IH but also on the gradual increase in ventilation to sustained hypercapnia was also considered by (Harris et al., 2006). In

their study, a subset of their population was exposed to sustained hypercapnia without exposure to hypoxia, but in contrast to our results, ventilation remained constant after baseline measurements suggesting that it was indeed acute IH that mediated ventilatory LTF. However, a more recent paper has demonstrated that ventilation can also be significantly elevated in OSA patients following a slightly longer but lower intensity of sustained elevation in PET_{CO_2} without hypoxic exposure (Gerst et al., 2011). The significant increase in \dot{V}_E due to hypercapnia alone in our study occurred even though we extended the duration of the hypercapnic acclimatisation phase by seven minutes from that used by (Gerst et al., 2011) in order to allow longer for ventilation to plateau following the step increase in PET_{CO_2} . The exact mechanism(s) responsible for this gradual rise in ventilation due to mild hypercapnia remains to be determined, although our data showing hyperoxia reduced ventilation to the same extent at baseline and recovery suggest a central mechanism is responsible.

Respiratory LTF following acute IH and acute CH

Despite our results demonstrating that there is a significant gradual increase in ventilation to sustained hypercapnia that is independent of hypoxic exposure (*Trial 3*), this increase is significantly less than that occurring following the addition of acute IH or acute CH (*Trial 1* and *Trial 2*). We believe these results demonstrate that in awake humans ventilatory LTF does manifest following exposure to both acute IH and acute CH during sustained hypercapnia albeit by a smaller magnitude

than previously documented. Furthermore, we have previously demonstrated that in awake humans ventilation was significantly greater during a brief five minute period of elevated P_{ETCO_2} (~1 mmHg above normal levels) following hypoxic airway occlusions vs. room air breathing. Therefore, the ventilatory LTF in chapter 4 could not have been mediated by hypercapnia *per se* as the duration and level of hypercapnia were the same in both cases.

Our findings are in contrast to animal research that suggests that intermittent but not continuous exposure to hypoxia can evoke respiratory LTF. Respiratory LTF following acute IH has been demonstrated in various anaesthetised and conscious animal species (Mateika and Sandhu, 2011) but not following acute CH (Baker and Mitchell, 2000, Mitchell et al., 2001b, Turner and Mitchell, 1997, Dwinell et al., 1997, McKay et al., 2004, Tadjalli et al., 2007).

The magnitude of ventilatory LTF following acute IH and acute CH in our study was equivalent and comparable to that of previous studies demonstrating ventilatory LTF following acute IH in healthy awake humans (Harris et al., 2006, Lee et al., 2009, Wadhwa et al., 2008). Small differences in the magnitude of ventilatory LTF between studies are inevitable due to differences in protocols such as the total number of hypoxic episodes, level of sustained CO_2 , duration of recovery and differences in the participant population. However, one noticeable difference worth discussing is that the time course of ventilatory LTF development following acute

IH and acute CH in our study is different to that previously demonstrated in studies following acute IH (Lee et al., 2009, Harris et al., 2006, Wadhwa et al., 2008, Gerst et al., 2011). In these previous studies, \dot{V}_E does not change during hypercapnic recovery but remains stable and elevated above baseline having progressively increased during each euoxic episode that follows hypoxic exposures. In contrast the increase in \dot{V}_E during euoxic episodes was more modest in our study, with only a noticeable increase following the first hypoxic exposure and then again during the final two hypoxic exposures. Furthermore, there was a gradual rise in \dot{V}_E during hypercapnic recovery following both acute IH and acute CH and thus \dot{V}_E was significantly greater during the final quarter of hypercapnic recovery compared with the first. No change in \dot{V}_E during hypercapnic recovery in *Trial 3* suggests the increase in \dot{V}_E during hypercapnic recovery following acute IH and acute CH is due to hypoxic exposure rather than a continued increase in \dot{V}_E to the sustained hypercapnia. Interestingly, OSA patients have been shown to both demonstrate (Lee et al., 2009) and not demonstrate this increase in \dot{V}_E (Gerst et al., 2011) during hypercapnic recovery. Although the pattern of increasing ventilatory LTF during hypercapnic recovery shown in our study is different to that of previous studies demonstrating ventilatory LTF development in healthy humans, this pattern is more common in animals that express respiratory LTF following acute IH. In anaesthetised rats phrenic and hypoglossal nerve and diaphragmatic muscle LTF as well as ventilatory LTF demonstrate a progressively augmenting pattern during

recovery from acute IH (Mitchell et al., 2001a, Ryan and Nolan, 2009a, Bach and Mitchell, 1996, Baker and Mitchell, 2000, Olson et al., 2001).

In addition to the expression of ventilatory LTF following acute IH the significant increase in \dot{V}_E during the last hypoxic episode compared with the initial exposure is indicative of progressive augmentation of the HVR. As previously discussed in detail, progressive augmentation of the HVR and ventilatory LTF may simply be the same form of respiratory plasticity and they are just demonstrated under different O_2 tensions. However, as was also concluded in chapter 4 where augmentation of the HVR was shown following hypoxic airway occlusions, we do not have enough evidence to support or refute this theory.

The role of the CB in maintaining ventilatory LTF

We hypothesised that CB afferent discharge during recovery from acute IH and acute CH is actively involved in maintaining ventilatory LTF in awake humans. In contrast to this hypothesis, hyperoxia reduced ventilation to a similar extent during hypercapnic baseline and recovery in both trials suggesting that the CB does not contribute to ventilatory LTF as shown by the increase in ventilation following acute IH and acute CH.

Our results suggest that mechanisms independent to the CB maintain ventilatory LTF in awake humans and accordingly, agree with a substantial body of evidence

from animals. The demonstration of respiratory LTF following acute IH during CB inhibition with hyperoxia as shown in our study has previously been demonstrated in rats (Bach and Mitchell, 1996, Baker and Mitchell, 2000, Xing and Pilowsky, 2010). Although these studies were not designed specifically to investigate the involvement of the CB in maintaining respiratory LTF, rats were exposed to 50% (Bach and Mitchell, 1996, Baker and Mitchell, 2000) or 100% (Xing and Pilowsky, 2010) inspired O₂ throughout baseline and recovery, which would be expected to inhibit CB afferent discharge. However, despite this, phrenic discharge was shown to increase progressively during recovery and remained elevated for > 1 hr. This does not preclude the possibility of a stimulus to the CB that arises during acute IH, but is *not* inhibited by hyperoxia. To date, however, there is no evidence of any such residual, oxygen-independent, component arising during acute IH. Of course, if such a stimulus did exist then a role for the CB cannot be excluded. In addition, the possibility of a necessary input from the CB in LTF development is further eroded by the finding that episodic stimulation of the cut carotid sinus nerve to mimic episodic activation of the CB during acute IH initiates phrenic LTF which is maintained for > 30-90 minutes, following the total absence of any CB input to the central nervous system (CNS) (Millhorn et al., 1980a, Hayashi et al., 1993).

Direct recordings of *in* and *ex vivo* CB afferent discharge in rats not previously exposed to acute IH demonstrated the elevated afferent discharge during hypoxic episodes promptly returned to baseline levels upon re-oxygenation and remained constant throughout recovery (Peng et al., 2003). However, rodents previously

exposed to chronic IH aimed to mimic nightly IH experienced by OSA patients evoked a progressive increase in CB afferent discharge during successive hypoxic episodes which stayed elevated for > 1 hr in recovery. This sustained elevation in CB afferent discharge following acute IH that is only evident in chronic IH rats has been termed sensory LTF and is reactive oxygen species dependant as antioxidant treatment inhibits its manifestation (Peng et al., 2003). All participants in our study reported no current or previous history of sleep apnoea and thus can be considered naïve to previous IH exposure. Our healthy participants would therefore not be expected to develop sensory LTF following acute IH. Using an almost identical acute IH protocol as used in our study, (Lee et al., 2009) demonstrated that OSA patients produced greater ventilatory LTF than healthy controls. Interestingly, antioxidant treatment attenuated this augmentation in ventilatory LTF back to the same level of ventilatory LTF as the healthy controls where antioxidant treatment had no effect. Presumably this was consequent to the abolishment of sensory LTF by antioxidant treatment in OSA patients that did not manifest in healthy controls who had previously not been exposed to chronic IH. However, the contribution that sensory LTF has on ventilatory LTF remains unknown and requires future studies utilising techniques such as hyperoxic inhibition of the CB.

CB afferent discharge may reach the presympathetic neurons without intermediate communication in the respiratory network (Guyenet, 2000). Thus, it is possible that augmented CB afferent discharge (sensory LTF) or enhanced integration of CB afferent discharge at the CNS could mediate sympathetic LTF as previously shown

by (Querido et al., 2010) but not mediate a parallel increase in ventilation (respiratory LTF). We did not record sympathetic activity in our study and therefore we cannot conclude whether the hyperoxic exposures that failed to attenuate ventilatory LTF also attenuated sympathetic LTF.

CB independent mechanisms maintaining ventilatory LTF in acute IH and acute CH

Our findings that the CB does not actively maintain respiratory LTF suggest that a central mechanism may be involved. Whilst there is no evidence to suggest that an increase in cerebral blood flow (CBF) due to relatively short, acute exposures to hypoxia and/or hypercapnia adapts towards control levels (Poulin et al., 1996) causing a relative accumulation of CO₂ in the cerebral circulation, a possible stimulus could be a lower pH in the cerebrospinal fluid caused by impaired regulation of [H⁺] during hypoxia (Duffin, 2005). Thus, ventilatory LTF may reflect the time course of central pH recovery following exposure to systemic hypoxia. Whilst we have no data to support or refute this possibility, this would be an important consideration for any mechanistic explanation of ventilatory LTF.

It has long been believed that ventilation is not simply an additive product of peripheral and central chemoreflex input and recently a fascinating study by (Blain et al., 2010) presented strong evidence demonstrating that CB afferent discharge expresses a substantial hyperadditive interaction on the ventilatory response to central hypercapnia in dogs. The authors suggest that this significant interaction

may influence acclimatisation to continuous hypoxia and also other clinical conditions such as altered ventilatory control following chronic IH in OSA. However, because hyperoxia did not inhibit the ventilatory LTF in our study we can be confident that the ventilatory LTF following acute IH and/or acute CH is not due to an enhancement of this hyperadditive interaction.

Animal studies provide evidence for a serotonin-dependent mechanism initiating and maintaining respiratory LTF (Mahamed and Mitchell, 2007, MacFarlane et al., 2008). Phrenic LTF requires spinal serotonin release (Baker-Herman and Mitchell, 2002) and activation of serotonin receptors (5-HT₂) on respiratory motoneurons in the spinal cord (Kinkead and Mitchell, 1999, Fuller et al., 2001) which initiate the synthesis of spinal proteins such as *brain-derived neurotrophic factor* (BDNF) that subsequently maintains phrenic LTF (Baker-Herman et al., 2004). As stimulation of the cut carotid sinus nerve initiates the same serotonergic-dependant pathway of phrenic LTF as acute IH (Millhorn et al., 1980b, Bach and Mitchell, 1996), the above model for serotonin-dependant respiratory LTF does not imply that the CB is not necessary for initiating respiratory LTF, rather that spinal protein synthesis maintains respiratory LTF as opposed to any continued input from the CB. Additionally, inhibition of 5HT₂ receptors prior to acute IH exposure but not immediately following acute IH abolished phrenic LTF (Fuller et al., 2001). Although this serotonin-dependant mechanism for acute IH-induced respiratory LTF has been well described in animal studies (Mahamed and Mitchell, 2007, MacFarlane et al., 2008), acute CH does not elicit the same pattern of respiratory

LTF (Baker and Mitchell, 2000, Mitchell et al., 2001b, Turner and Mitchell, 1997, Dwinell et al., 1997, McKay et al., 2004) and only intermittent but not continuous application of serotonin or 5-HT₂ receptor agonists evokes phrenic LTF (Lovett-Barr et al., 2006, MacFarlane and Mitchell, 2009) and hypoglossal LTF (Bocchiaro and Feldman, 2004). Therefore, animal studies can only provide us with limited clues as to the possible mechanism(s) for ventilatory LTF following acute CH in awake humans as shown in our study. However, a recent study reported an equivalent magnitude and pattern of phrenic LTF following acute CH to that of acute IH, when serine/threonine protein phosphatase in the spinal cord of rats exposed to acute CH was inhibited with okadaic acid (Wilkerson et al., 2008). Furthermore, in the same study intravenous infusion of the broad spectrum serotonin inhibitor, methysergide, blocked phrenic LTF in rats exposed to acute IH or acute CH with okadaic acid. Okadaic acid administration alone did not evoke phrenic LTF and these results thus suggest the equivalent phrenic LTF following acute IH and acute CH with okadaic acid were mediated by the same serotonin-dependant mechanism. Reactive oxygen species (ROS) are known to inhibit many protein phosphatases and observations from various studies would suggest that acute IH-induced ROS may inhibit these okadaic acid-sensitive serine/threonine phosphatases and thus remove their inhibitory restraint on phrenic LTF which does not occur during acute CH a stimulus not known to induce ROS generation (Wilkerson et al., 2007). Indeed, MacFarlane and Mitchell (2009) showed intermittent serotonin application to the spinal cord induces phrenic LTF but was abolished with NADPH oxidase inhibition (MacFarlane and Mitchell,

2009)(MacFarlane and Mitchell, 2009). An attractive explanation for the appearance of ventilatory LTF following acute CH in our study is that the existence or regulation of these specific okadaic acid-sensitive serine/threonine phosphatases during hypoxic exposure is different in rats and thus the serotonin-dependant mechanism for respiratory LTF during acute CH is not constrained in humans.

Although serotonin-induced phrenic LTF (Lovett-Barr et al., 2006, MacFarlane and Mitchell, 2009) and hypoglossal LTF (Bocchiaro and Feldman, 2004) require intermittent activation of serotonin receptors, different respiratory pools may not require an intermittent pattern. Indeed, continuous spinal serotonin application is sufficient to induce LTF in thoracic (intercostals) inspiratory motor output in the neonatal rat brainstem/spinal cord preparation (Lovett-Barr et al., 2006). As ventilation is a product of combined respiratory output from various respiratory motor pools it is plausible that significant thoracic LTF following acute CH could in part mediate the ventilatory LTF demonstrated in our study. Furthermore, humans may demonstrate a variation from the situation in the rat in which respiratory motor pools express serotonin-induced LTF to continuous activation of serotonin receptors and thus phrenic LTF or hypoglossal LTF may be evoked by acute CH.

If hypoxic-induced CB activity was the only mechanism capable of initiating respiratory LTF then CB denervation should completely abolish respiratory LTF when in fact it has been shown in rats to only attenuate acute IH-induced phrenic

LTF (Sibigtroth and Mitchell, 2011, Bavis and Mitchell, 2003). Therefore, although phrenic LTF following acute IH and episodic stimulation of the carotid sinus nerve is completely abolished with methysergide (Bach and Mitchell, 1996, Millhorn et al., 1980b) suggesting a serotonin-dependant mechanism is capable of mediating the full expression of phrenic LTF in rats with intact CBs, it would appear that it is a redundant system as an additional central mechanism(s) must exist for phrenic LTF to develop in CB denervated rats. The exact central mechanism(s) remains unknown but in the absence of chemoreceptor feedback from the CB during hypoxia the initiating stimulus must be central hypoxia. It is therefore possible that central hypoxia may have been responsible for part or all of the ventilatory LTF following acute IH and more likely following acute CH where the 32 minutes of sustained hypoxia may have allowed greater development of central hypoxia than during the four-minute episodes in acute IH.

A further intriguing possibility is that the ventilatory LTF following acute IH and acute CH in our study represents two different forms of respiratory LTF. The majority of animal studies investigating respiratory LTF use anaesthetised rats and demonstrate a progressive increase in phrenic LTF or ventilatory LTF following acute IH that does not peak until > 30 minutes and is sustained for > 1hr (Mitchell et al., 2001a). Ventilatory LTF following acute IH in awake unrestrained rats follows an equivalent pattern to anaesthetised rats (Olson et al., 2001). However, under these awake and unrestrained conditions that most closely mimic the experimental conditions of our study, acute CH evoked a significant sustained elevation in

ventilation that peaked immediately following acute CH and then declined back to baseline levels within 40 minutes. It was suggested that the differences in the pattern of the elevated ventilation following acute IH and acute CH might arise as they represent different forms of LTF (Olson et al., 2001). In contrast, there did not appear to be noticeable differences in the pattern of the elevated ventilation following acute IH and acute CH in our study. It is possible that differences would have been displayed if our relatively short recovery period was extended.

Summary

We have demonstrated that, in awake humans, acute CH evokes an equivalent magnitude and pattern of sustained elevation in ventilation as that of acute IH and that part of this apparent ventilatory LTF is due to a gradual ventilatory 'drift' induced by the concomitant hypercapnia. Furthermore, by demonstrating that CB inhibition with hyperoxic exposure does not attenuate ventilatory LTF we provide further corroborative evidence against a role for the CB in actively maintaining ventilatory LTF.

6 CHAPTER 6 - USE OF AN ANIMAL AIRWAY OCCLUSION MODEL OF OSA TO INVESTIGATE WHETHER SIMULTANEOUS HYPERGLYCAEMIA AUGMENTS IH-INDUCED OXIDATIVE STRESS

6.1 Introduction

Elevations in biomarkers of oxidative stress have been reported in the plasma (Christou et al., 2009) and urine (Yamauchi et al., 2005) of OSA patients and are correlated to hypoxic indices such as, the average nadir in S_aO_2 and the frequency of O_2 de-saturation events. Furthermore, exposure to acute IH evokes increased oxidative stress in OSA patients (Lee et al., 2009) and healthy humans (Pialoux et al., 2009). In addition, animals exposed to chronic IH demonstrate elevated levels of oxidative stress and develop cardiovascular disease which are both attenuated by antioxidant treatment suggesting oxidative stress as causal in the development of cardiovascular disease (Burckhardt et al., 2008, Troncoso Brindeiro et al., 2007, Peng et al., 2006). Another disease where oxidative stress is heavily implicated in the mediation of health complications is T2DM. Chronic hyperglycaemia-induced ROS generation in T2DM patients is believed to play a major role in the mediation of many of the associated health complications of T2DM (e.g. blindness, renal failure, nerve damage and atherosclerosis) (Brownlee, 2001). Furthermore,

experimentally induced chronic hyperglycaemia even without coexisting comorbidities (e.g. obesity) has been shown to elevate levels of oxidative stress in animal studies (Kayali et al., 2003, Cakatay and Kayali, 2006). In contrast, acute exposure to hyperglycaemia in insulin sensitive humans and rodents does not appear to cause an elevation in oxidative stress (Fisher-Wellman and Bloomer, 2010, Kayali et al., 2003, Cakatay and Kayali, 2006). However, although acute exposure to hyperglycaemia may not independently increase oxidative stress there is a line of evidence that has lead us to hypothesise that acute hyperglycaemia may greatly augment the degree of oxidative stress occurring during exposure to IH. As previously discussed in detail (General introduction: *Mechanisms for IH-induced oxidative stress*) the mechanistic pathways responsible for the generation of ROS during IH and IRI may be similar because of the comparable pattern of hypoxia and re-oxygenation (Lavie, 2003, Pack and Gislason, 2009). Recent evidence from experimental animal studies has shown that acute hyperglycaemia augments the generation of ROS and oxidative stress during exposure to IRI (Hirose et al., 2008, Bemeur et al., 2007, Tsuruta et al., 2010). Indeed, in two of these studies acute hyperglycaemia *per se* had no effect on ROS generation or oxidative stress but greatly augmented the response during both ischaemia and early reperfusion (Hirose et al., 2008, Tsuruta et al., 2010). We believe research is required to investigate whether these findings in IRI are also true for IH because over half of T2DM patients suffer from OSA (Einhorn et al., 2007) and thus millions of patients worldwide will experience hyperglycaemia and IH simultaneously during sleep.

Previously (chapter 3 and 4) we have used a newly designed airway occlusion model of OSA in healthy awake humans to investigate the effects of IH that occurs in OSA. Although we felt this model was successful in closely mimicking the generation of IH that occurs during obstructive apnoeas in OSA, participants found performing the airway occlusions for long periods uncomfortable. Therefore, this model is not suitable for exposing individuals to airway occlusions for a similar duration to that experienced by OSA patients during sleep (i.e. 6-8 hours). An alternative approach is to use an animal airway occlusion model of OSA whereby longer exposures are feasible. Indeed, it is possible to frequently occlude an airway cannula in anaesthetised rodents evoking transient but significant drops in PaO_2 that mimic those occurring in OSA (Simpson et al., 2008, Othman et al., 2010, Tadjalli et al., 2010). We therefore feel this is a suitable model to use in order to investigate our hypothesis that simultaneous hyperglycaemia will increase the magnitude of oxidative stress elicited by IH.

In addition to investigating this primary hypothesis using an animal model of airway occlusions we are also able to gain an insight into the possible development of ventilatory LTF and progressive augmentation of the HVR in OSA. Although airway occlusions in OSA evoke a significant magnitude of IH that would be expected to evoke ventilatory LTF and progressive augmentation of the HVR, to date there exists no conclusive evidence confirming or refuting the existence of these

phenomena in OSA. The absence of any evidence is because of the difficulty in accurately measuring a change in ventilatory control in OSA patients for two main reasons. Firstly, in most OSA patients there is an absence of suitable durations of unrestricted breathing to assess ventilation during room air breathing or hypoxic exposures and secondly changes in sleep stage elicit rapid changes in ventilation which may confound results.

In chapter 4 we exposed healthy individuals to hypoxic airway occlusions aimed to mimic OSA and were able to demonstrate the expression of ventilatory LTF and progressive augmentation of the HVR but only when PET_{CO_2} was elevated by ~1 mmHg above normal levels. It has previously been hypothesised that the demonstration of respiratory LTF and progressive augmentation of the HVR in awake humans when PET_{CO_2} is raised above normal levels is because ventilation is then controlled by the central and peripheral chemoreflex, rather than arousal and/or behavioural stimuli (Harris et al., 2006). As ventilation in anaesthetised animals cannot be maintained by arousal and/or behavioural stimuli it would seem likely that airway occlusions that elicit a significant magnitude of IH would evoke ventilatory LTF and progressive augmentation of the HVR. Although a number of studies with anaesthetised or sleeping animals have been undertaken previously to investigate the effects of repetitive airway occlusions (Simpson et al., 2008, Othman et al., 2010, Tadjalli et al., 2010, Schoorlemmer et al., 2011) only one investigated whether ventilatory LTF manifest (Tadjalli et al., 2010). In this study

rats who were exposed to a ten minute protocol of 15 second airway occlusions once every minute did not demonstrate LTF of diaphragm activity. However, we considered it possible that the small number of airway occlusions used in this study may have been insufficient to elicit ventilatory LTF.

Study aims

The primary aim of this study was to assess whether simultaneous exposure to hyperglycaemia augments the level of oxidative stress in animals exposed to repetitive airway occlusions that replicate IH occurring in OSA. A secondary aim of the study was to investigate whether ventilatory LTF and progressive augmentation of the HVR manifest in animals exposed to airway occlusions under poikilocapnic conditions for a similar duration to that experienced by OSA patients.

6.2 Methods

Experiments were performed on 24 male Wistar rats (weight 279 ± 57 g; Charles River, Kent, UK) (mean \pm standard deviation). All rats were housed in cages in the Biomedical Services Unit (BMSU) at the University of Birmingham and kept on a controlled 12 hour light-dark cycle and fed standard rat chow and water *ad libitum*. Food was withdrawn from rats at 4pm the afternoon before experiments and replaced with a food ration of 10g standard rat chow so all rats were considered

fasted at the start of each experiment. All procedures complied with the Animals (Scientific Procedures) Act 1986.

Anaesthesia

Anaesthesia was induced in a small volume chamber via inhalation of isoflurane (3-4 %) in 3-4 l/min O₂. Animals were deemed sufficiently anaesthetised upon a lack of righting ability and loss of the pedal withdrawal reflex at which time they were immediately placed on an operating table in the supine position and anaesthesia maintained via a face mask with 3-4 % isoflurane in 3-4 l/min O₂. A vinyl cannula (Portex OD = 1.40 mm, ID = 0.63 mm) was then inserted into the jugular vein so that anaesthesia could be maintained with the steroid anaesthetic alfaxan (alfaxalone 10 mg/ml dissolved in cyclodextrin) diluted 1:1 with 0.9% saline. The flow of isoflurane was stopped and the face mask removed. Anaesthesia was then maintained throughout the experiment by a continuous infusion of alfaxan (~1.2 ml/h; dose of ~21.5 mg/kg/h) via an infusion pump (Braun Perfusor Secura FT, Braun, Germany). Somatic reflexes were frequently tested throughout the experiment in order to determine the depth of anaesthesia and additional bolus doses of 0.05 ml alfaxan were administered if deemed necessary.

Surgical Preparation

Following initiation of the continuous infusion of alfaxan the trachea was isolated and cannulated with a stainless steel T-shaped cannula. The T-shaped cannula maintained patency of the airway and allowed control of inspired gases using a

rotameter (e.g. hypoxia or room air) and allowed the airway to be transiently occluded as described below. A fluid filled cannula connected to a pressure transducer was introduced into the steel cannula and was sealed to allow airway pressure to be continuously measured. A short length of Nalgene tubing was attached to the remaining external end of the stainless steel T-shaped cannula which could be rapidly collapsed by a solenoid valve (2-way no pinch valve Kinesis Ltd, Cambridge, UK) to induce upper airway occlusions (see: *experimental protocols*). Immediately beyond the solenoid a spirometer was attached to measure airflow. Hypoxia could be induced by flowing 10% O₂ balance nitrogen past the spirometer from a rotameter.

The right brachial artery was cannulated with polythene tubing (Portex, OD = 0.8 mm, ID = 0.4 mm) and was used to draw arterial blood samples for the assessment of blood glucose concentrations and blood gases.

The right femoral artery was cannulated with polythene tubing (Portex, OD = 0.96 mm, ID = 0.58 mm) and BP was continuously measured with a pressure transducer. Prior to each experiment the pressure transducer was calibrated with a mercury sphygmomanometer.

The femoral vein was isolated and cannulated with vinyl cannula (Portex OD = 1.40 mm, ID = 0.63 mm) and used for the infusion of glucose or saline.

The animal was maintained at 37°C throughout the experiment using a thermostatic blanket that automatically corrected the temperature based on a thermocouple that measured rectal temperature.

A MacLab/8s (AD Instruments Ltd, Oxford, UK) data acquisition system sampling at 100 Hz was used to collect cardiovascular and respiratory variables onto a Power Mac G4 computer with Chart software (AD Instruments Ltd.). Mean arterial pressure (MAP) was calculated on-line from the BP trace and TV and breathing frequency were derived on-line using the continuous air flow trace. \dot{V}_E was calculated off line by multiplying the two respiratory components. To determine the S_aO_2 during airway occlusions a pulse oximeter was attached to a paw of three rats in the NGAO group and S_aO_2 immediately prior to airway occlusions and the nadir following airway occlusions was manually recorded.

Following surgery and setting up all recording equipment 20-30 minutes of equilibration was given before a baseline blood glucose measurement was taken once all measurements were deemed stable. Blood glucose was measured from a small drop (~0.6 μ l) of arterial blood using an AccuChek Aviva Glucose Meter (Roche Diagnostics GmbH, Mannheim, Germany). In addition, in four animals arterial blood samples (150 μ l) were removed anaerobically and $PaCO_2$, PaO_2 and pH was assed using a blood gas analyser (Instrumentation Laboratory, MA, USA). This was repeated again during baseline hypoxia and room air recovery.

At the end of each experimental protocol blood was drawn from the femoral artery and transferred into a 5 ml K3-EDTA vacutainer (Becton Dickinson) previously kept on ice. Blood was then immediately centrifuged at 2,000g at 4°C for 12 minutes. Plasma was then aspirated into several aliquots that were precooled on ice and immediately frozen in liquid nitrogen before being moved to storage at –80°C. At the end of each experiment the animal was killed by exsanguination.

Experimental protocol

Male, Wistar rats were investigated in this study in four different groups.

Group 1 (time control): Normal glucose airway unrestricted - (NGAU)

Baseline measurements were recorded for 10 minutes during room air ventilation. Following this, animals were exposed to three minutes of poikilocapnic hypoxia (10% inspired O₂). A further 5-10 minutes of room air ventilation was given to ensure blood gases had returned to normal and all measurements had stabilised before a saline bolus (10 ml/kg) was administered. Immediately following the saline bolus a constant infusion of saline was started at an infusion rate of 0.6-0.8 ml/h (chosen to approximately match the volume infused in rats in the two hyperglycaemic groups). Blood samples were drawn and assessed for blood glucose levels every half hour for six hours. After six hours, recovery measurements were performed which were identical to those performed at baseline (i.e. ten minutes of room air breathing followed by three minutes of

poikilocapnic hypoxia). Upon completion of recovery measurements, saline infusion was stopped and > 5 ml of blood was drawn for later assessment of oxidative stress and antioxidant capacity.

Group 2: High glucose airway unrestricted - (HGAU)

Animals in group 2 were exposed to a similar experimental exposure as those in group 1 except they were additionally exposed to sustained hyperglycaemia. The saline bolus was replaced with a glucose bolus (50 % w/v in saline; Sigma Aldrich, Poole, UK) of (10 ml/kg) and glucose was then infused at a rate selected with the aim of maintaining blood glucose levels between 11-12 mM/l for the remainder of the experiment (see: figure 6.1 for average infusion rates). Blood samples were drawn and assessed for blood glucose levels every half hour for six hours. If required glucose infusion rate was adjusted following each assessment of blood glucose levels. Baseline and recovery measurements were identical to those performed in group 1.

Group 3: Normal glucose airway occlusion - (NGAO)

Animals in group 3 were exposed to the same saline bolus and saline infusion as described in group 1 but were also exposed to six hours of airway occlusions. Upon initiating the saline infusion animals were simultaneously exposed to airway occlusions aimed to mimic a pattern of IH that occurs in OSA. Using an automated system (Master-8 stimulator) to control the solenoid valve, the airway cannula was

occluded for fifteen seconds every minute for six hours. Baseline and recovery measurements were identical to those performed in group 1.

Group 4: High glucose airway occlusion - (HGAO)

Animals in group 4 were exposed to hyperglycaemia as described in group 2 simultaneously to airway occlusions as described in group 3 (i.e. six hour exposure to airway occlusions beginning immediately following the glucose bolus). Baseline and recovery measurements were identical to those performed in group 1.

Analytical procedures

MDA: A commercially available ELISA kit was used for the assessment of malondialdehyde-protein adducts (MDA) in plasma (OxiSelect MDA Adduct; Cell Biolabs, San Diego, CA).

MDA assay principle: In brief, MDA-protein adducts were expressed relative to protein concentration thus the total plasma protein concentration was first determined using the bicinchoninic acid method (BCA) as previously described by Smith *et al.* (1985). Six protein standards were prepared by diluting a stock solution of Bovine Serum Albumin (BSA) (1 mg/ml) in dH₂O. Standards (10 µl) and samples (10 µl, diluted 1:200 in dH₂O) were added to a 96 well plate in triplicate and 200 µl of BCA working solution (250 µl of copper sulphate solution (4% w/v) to 12.5 ml BCA solution (Sigma, UK)) added to each well. Plates were incubated at

37 °C for 30 minutes whilst protected from light. Plates were read at 490 nm (Multiscan MS, Labsystems, Finland) and total plasma protein concentration expressed as mg/ml.

Based on the BCA data plasma samples were diluted to 10 µg/ml in PBS and then diluted protein samples and standards were added to a 96 well protein binding plate. Rabbit antibody specific to MDA-protein adducts was added to the plate followed by a horseradish peroxidase (HRP) secondary antibody. Plates were read at 450 nm (Multiscan MS, Labsystems, Finland) and the concentration of MDA-protein adducts in the samples were determined by comparison with the standard curve and expressed as pmol/mg of protein.

Nitrotyrosine: A commercially available ELISA kit was used for the assessment of nitrotyrosine in plasma (Hbt nitrotyrosine; HyCult Biotechnology, Uden, Netherlands).

Nitrotyrosine assay principle: In brief, samples and standards were added to a 96 well plate coated in an antibody specific to nitrotyrosine. A biotinylated tracer antibody was then added which binds to the captured nitrotyrosine. Streptavidin-peroxidase conjugate was then added and binds to the biotinylated tracer antibody. Finally, tetramethylbenzidine was added which reacts with the enzyme streptavidin-peroxidase conjugate emitting a colour change. Plates were read at 450 nm (Multiscan MS, Labsystems, Finland) and the concentration of nitrotyrosine

in the samples were determined by comparison with the standard curve and expressed as nM.

FRAP: Total antioxidant capacity was assessed in plasma using a modification of the Ferric Reducing Ability of Plasma (FRAP) assay described previously by (Benzie and Strain, 1999). 10 µl of samples and 10 µl of standards (0-1000 µM ascorbic acid) were added in triplicate to a 96 well plate. 300 µl of freshly prepared FRAP reagent (300 mM acetate buffer (pH 3.6), 20 mM ferric chloride, 10 mM 2, 4, 6- tripyridyltriazine) was added and incubated for eight minutes at room temperature. Absorbance values were read at 650 nm (Multiscan MS, Labsystems, Finland) and expressed as µM of antioxidant power relative to ascorbic acid as determined from a seven-point linear standard curve.

Statistical analysis

One-way ANOVA in conjunction with a *post hoc* LSD test was used to determine whether there was a significant difference in oxidative stress (MDA, nitrotyrosine) and antioxidant capacity (FRAP) between the four groups of animals. A two-way mixed-design ANOVA in conjunction with a *post hoc* LSD test was used to determine whether there was a significant group-by-time interaction in $\dot{V}E$. Analysis was performed separately for room air conditions (i.e. baseline room air vs. recovery room air) and for hypoxic exposures (i.e. baseline hypoxia and recovery hypoxia). A one-way ANOVA with repeated measures was used to more closely

determine whether there was a change over time in \dot{V}_E in each group. All data are expressed as mean \pm SEM. Differences were considered significant if $P \leq 0.05$.

6.3 Results

Airway occlusion induced reductions in S_aO_2

Fifteen second airway occlusions caused significant hypoxia as demonstrated by an average decrease in S_aO_2 from 92.4 ± 0.38 % pre airway occlusion to 67.9 ± 2.3 % post airway occlusion in three rats in the LGAO group.

Blood gases

Arterial blood gas samples were assessed during baseline euoxia, baseline hypoxia and recovery euoxia in three rats in the NGAO group and one rat in the HGAO group. Pa_{CO_2} fell from 45.5 ± 1.5 mmHg during room air baseline to 42.8 ± 2.1 mmHg during room air recovery. This was accompanied by a change in pH which increased from 7.42 ± 0.03 during room air baseline to 7.45 ± 0.01 during room air recovery. Pa_{O_2} increased from 74.3 ± 3.2 mmHg during room air baseline to 81.5 ± 1.8 mmHg during room air recovery. Furthermore, exposure to 10% inspired O_2 induced a notable degree of hypoxia as Pa_{O_2} averaged 38.8 ± 2.7 mmHg. Subsequent hyperpnoea caused hypocapnia as Pa_{CO_2} averaged 31.5 ± 1.85 mmHg which was accompanied by an increase in pH which averaged 7.51 ± 0.01 .

Blood glucose

Figure 6.1 shows blood glucose values at baseline and every 30 minutes for six hours following glucose (HGAO and HGAU) or saline infusions (NGAO and NGAU) and also shows the glucose infusion rates (HGAO and HGAU). Blood glucose levels remained relatively stable throughout the experimental protocol in the NGAU and NGAO groups. In contrast, by design, blood glucose was elevated above baseline levels during glucose infusions in animals in the HGAU and HGAO groups. After an initial over shoot in the desired blood glucose levels in the first 30 minutes in both groups, blood glucose was reduced and stabilised at the desired value, averaging 11.6 ± 0.12 and 11.52 ± 0.14 mM for the remainder of the experiment for HGAU and HGAO groups respectively.

Oxidative stress and antioxidant capacity

Figure 6.2 shows plasma MDA levels for the four different groups of animals, where none of the groups demonstrated a significant difference from another. Figure 6.3 shows plasma nitrotyrosine for all four groups of animals. For the majority of animals plasma levels of nitrotyrosine was low and only slightly above the detection limit of the assay. However, there were also animals that exhibited more notable levels of plasma nitrotyrosine but as these animals were spread between all four groups there was no significant difference between groups. Figure 6.4 shows plasma antioxidant capacity as assessed by the FRAP assay. As with

the biological markers of oxidative stress there was no notable difference in antioxidant capacity between the four groups of animals.

Ventilation

Figure 6.5 shows averaged \dot{V}_E during the final three minutes of baseline and recovery room air breathing in all groups. \dot{V}_E significantly increased from baseline to recovery in the HGAU trial ($P = 0.016$) without a significant change in any of the other groups. However the change in \dot{V}_E over time in the HGAU group was not significantly different to the other groups. Figure 6.6 shows averaged \dot{V}_E for the first 20 seconds of the hypoxic exposure at baseline and recovery in all groups. \dot{V}_E did not change between baseline and recovery in any of the groups.

Mean arterial pressure

Figure 6.7 shows averaged MAP during baseline and recovery room air breathing in all four groups. None of the groups demonstrated a notable change over time.

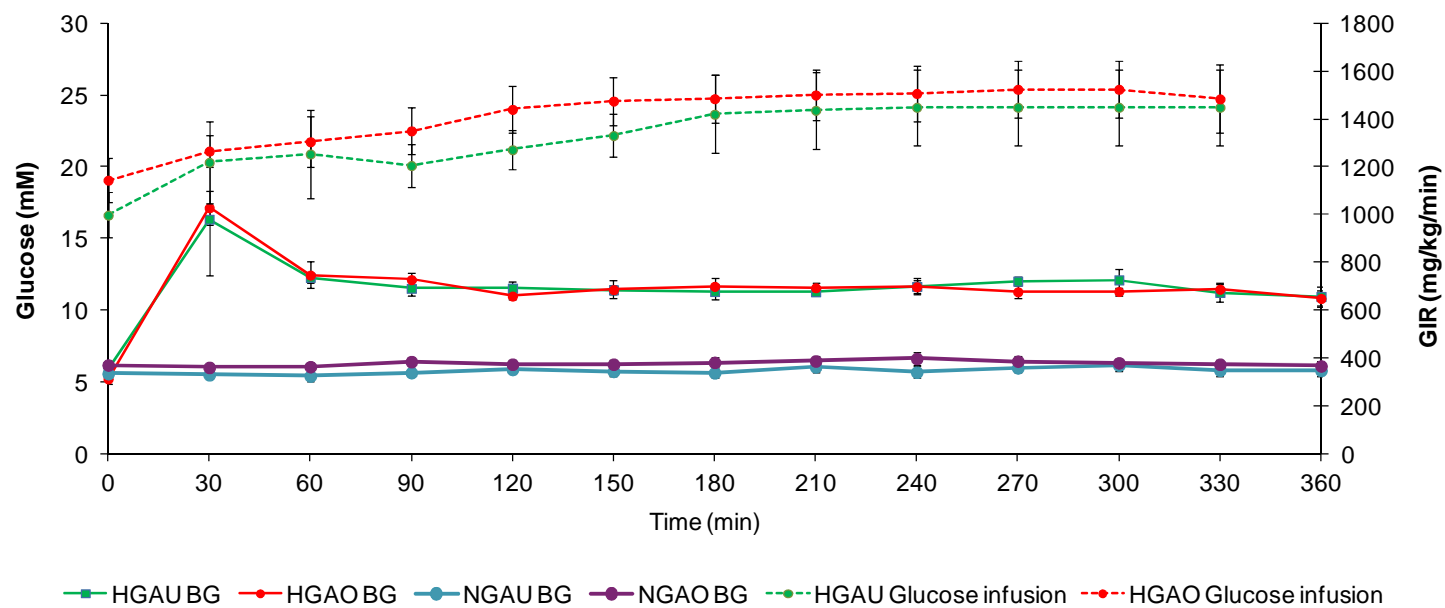


Figure 6.1: Solid lines indicate average blood glucose levels for all four groups taken at baseline (0 min) and every half hour for the next six hours (HGAO - red, HGAU - green, NGAO - purple, NGAU - blue). Dashed lines indicate glucose infusion rates (GIR) (HGAO - Red, HGAU - Green) with each point indicating the GIR set for the following half hour.

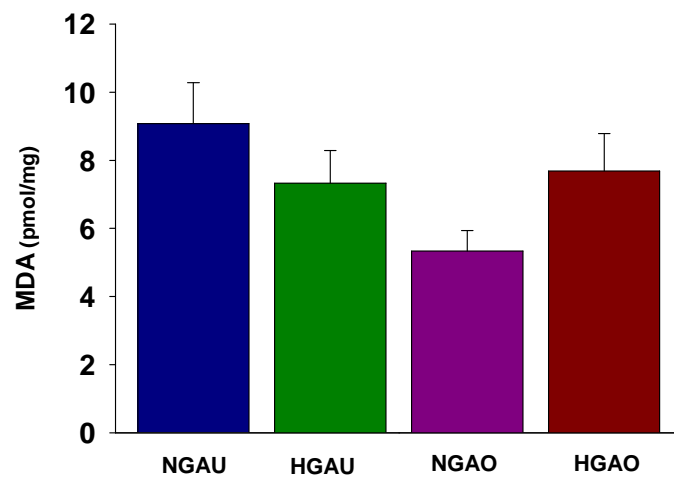


Figure 6.2: Averaged plasma levels of malondialdehyde-protein adducts (MDA) in all four groups of animals.

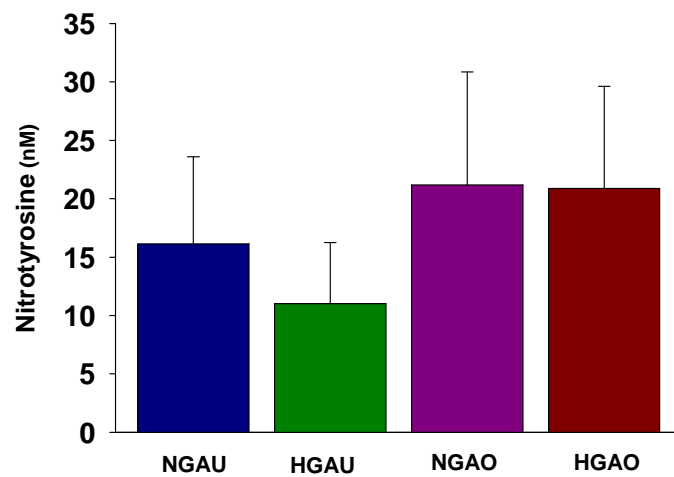


Figure 6.3: Averaged plasma levels of nitrotyrosine in all four groups of animals.

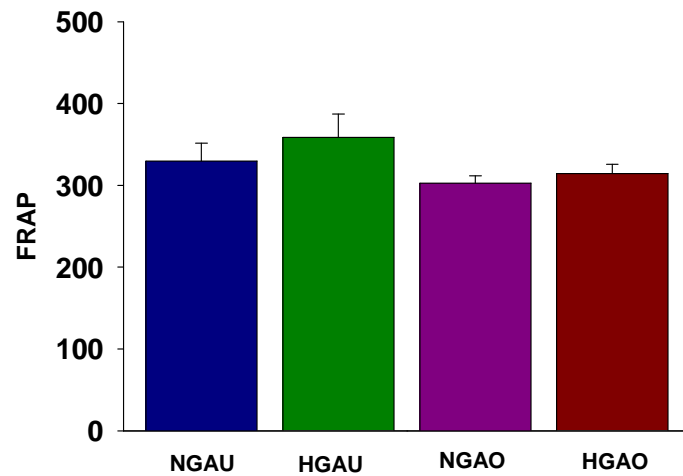


Figure 6.4: Averaged antioxidant capacity of plasma (FRAP) in all four groups of animals. (antioxidant power relative to ascorbic acid).

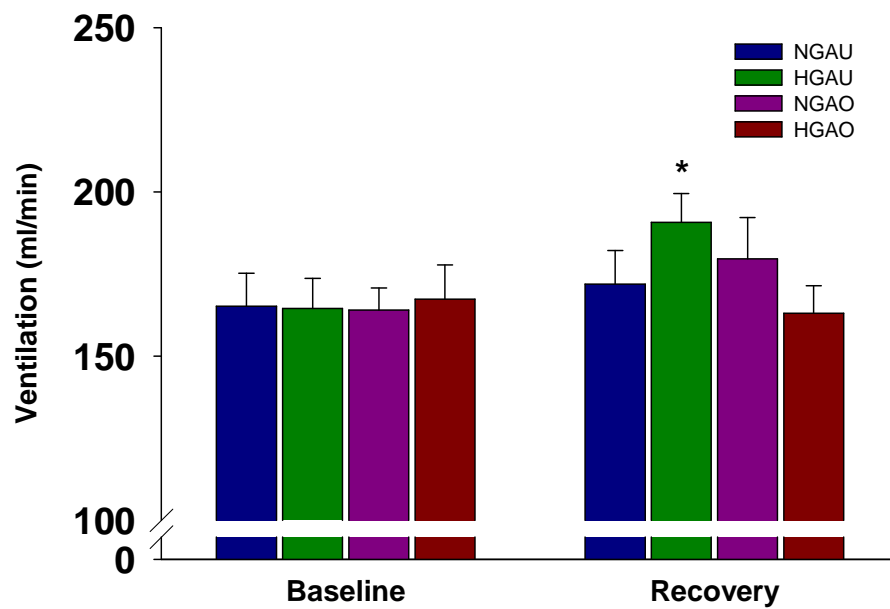


Figure 6.5: Averaged minute ventilation during baseline and recovery room air in all four groups of animals. * significantly greater than baseline $P < 0.05$.

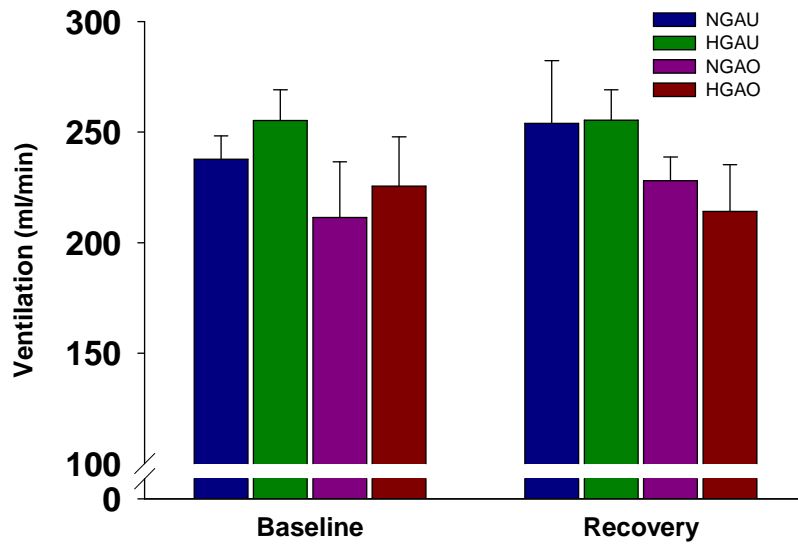


Figure 6.6: Averaged minute ventilation during the first twenty seconds of hypoxic exposure at baseline and recovery in all four groups of animals.

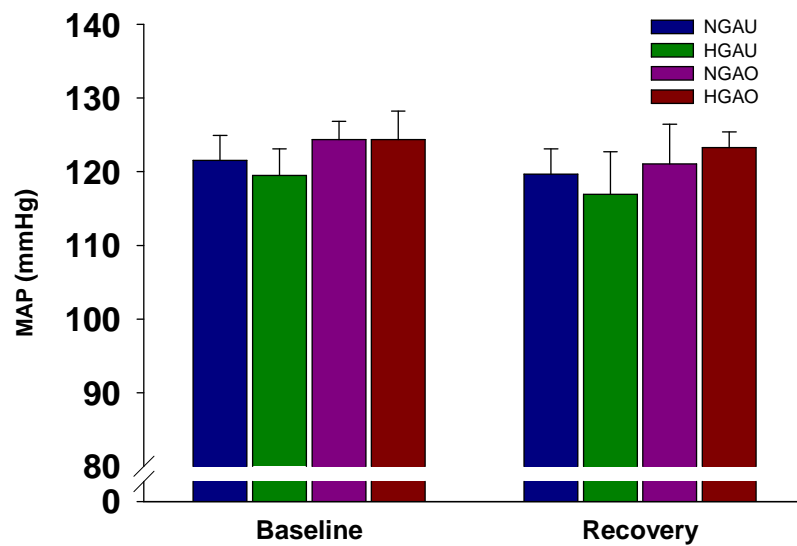


Figure 6.7: Averaged mean arterial pressure (MAP) during baseline and recovery room air breathing in all four groups of animals.

6.4 Discussion

The primary aim of this study was to determine whether exposure to simultaneous hyperglycaemia augmented the level of oxidative stress during exposure to repetitive airway occlusions that replicated the generation of IH occurring in OSA. Analysis of plasma levels of oxidative stress (MDA and nitrotyrosine) was unable to support our hypothesis as there was no notable elevation in oxidative stress in animals exposed to simultaneous (HAGO) rather than independent exposure to hyperglycaemia (HGAU) or repetitive airway occlusions (NGAO). Furthermore, the level of oxidative stress in these three groups of animals did not appear to be greater than in control animals (i.e. freely breathing, saline infused animals). Thus, suggesting that neither stimulus alone or simultaneously was capable of inducing oxidative stress in the healthy rats used in this study.

The secondary aim of this study was to investigate whether ventilatory LTF and/or progressive augmentation of the HVR manifest in rats exposed to six hours repetitive airway occlusions aimed to mimic a night of OSA. Ventilation during recovery room air breathing following six hours airway occlusions with or without simultaneous hyperglycaemia was not greater than during baseline room air breathing. In addition, there was no difference in the level of ventilation during baseline hypoxia and recovery hypoxia. In contrast, ventilation during recovery

room air breathing was significantly greater than baseline room air breathing in rats exposed to sustained hyperglycaemia without airway occlusions (HGAU).

Oxidative stress

Lipid peroxidation (LPO) represents oxidative damage of lipids most commonly associated with polyunsaturated fatty acids and elevated levels of LPO have been reported in plasma and tissues from patients with cardiovascular disease (Niki, 2009, Rumley et al., 2004). Peroxidation of polyunsaturated fatty acids generates a number of secondary products of which MDA is the major product. MDA expresses a high level of toxicity, and is able to alter DNA and various proteins and is considered to have atherogenic and mutagenic properties (Del Rio et al., 2005). Eight weeks of nightly IH exposure has been shown to cause a twofold increase in plasma MDA levels in rats (Savransky et al., 2007). Furthermore, simultaneous exposure to acute hyperglycaemia increased the generation of MDA in the plasma and brain of rodents exposed to cerebral IRI (Tsuruta et al., 2010). Therefore, we felt plasma MDA was an appropriate marker of oxidative stress to use in order to investigate whether simultaneous hyperglycaemia increased oxidative stress during repetitive airway occlusions aimed to replicate IH occurring in OSA. However, we were unable to demonstrate an appreciable difference in plasma MDA levels between any of the groups and control animals.

Oxidative stress is a product of ROS and reactive nitrogen species (RNS) of which peroxynitrite is one of the most potent RNS generated when superoxide anions

react with nitric oxide. Peroxynitrite can react with tyrosine residues in proteins forming a 3-nitrotyrosine adduct (Souza et al., 2008) and thus 3-nitrotyrosine is commonly used as a marker for peroxynitrite (Beckman and Koppenol, 1996, Oldreive and Rice-Evans, 2001). Nitrotyrosine has been shown to be associated with endothelial dysfunction (Kohler et al., 2010) likely as a result of increased levels of peroxynitrite reducing the bio-availability of nitric oxide. It has previously been demonstrated that OSA patients have elevated levels of nitrotyrosine in endothelial cells and impaired endothelial function as assessed by flow mediated dilation (FMD) (Jelic et al., 2010, Jelic et al., 2008). Furthermore randomized trials with CPAP have demonstrated improvements in circulating levels of nitrate and nitrite (Cross et al., 2008). Interestingly, it has also been shown in rodents that hyperglycaemia simultaneous to IRI increases nitrotyrosine in hepatic (Behrends et al., 2010), kidney (Hirose et al., 2008) and cerebral tissue (Ste-Marie et al., 2001). Therefore, we felt assessing the level of plasma nitrotyrosine would be an appropriate measurement to investigate whether simultaneous hyperglycaemia increased oxidative stress during airway occlusions. However, as with the assessment of plasma MDA levels we were unable to demonstrate a noticeable elevation in nitrotyrosine in any of the groups compared to control animals.

With the important role that antioxidants play in attenuating oxidative stress and evidence demonstrating impaired antioxidant capacity in disease states (Barcelo et al., 2006, Baynes, 1991) we sought to assess antioxidant capacity in addition to measures of oxidative stress. We used a FRAP assay to assess total antioxidant

capacity of the plasma which includes the sum of all antioxidants (i.e. endogenous and exogenous) (Bartosz, 2010). Having demonstrated both separate and simultaneous exposure to hyperglycaemia and airway occlusions did not increase the level of oxidative stress in animals it is maybe not surprising that there was no difference in the antioxidant capacity of the plasma between groups.

We did not expect oxidative stress to be elevated in animals exposed to acute hyperglycaemia alone as previous literature has shown that acute exposure to hyperglycaemia does not increase oxidative stress in insulin sensitive rodents (Cakatay and Kayali, 2006, Kayali et al., 2003) or healthy humans (Fisher-Wellman and Bloomer, 2010). However, we did expect to see an increase in oxidative stress following six hours of airway occlusions that evoked a significant degree of IH, because of existing human research that demonstrates acute exposure to experimentally-induced IH causes oxidative stress (Pialoux et al., 2009, Lee et al., 2009). Furthermore, we hypothesised that simultaneous hyperglycaemia would enhance IH-induced oxidative stress as it has been shown to do during IRI in various animal studies (Hirose et al., 2008, Bemeur et al., 2007, Tsuruta et al., 2010). Although elevated levels of plasma MDA have been shown in animals exposed to chronic IH (Savransky et al., 2007), the study does not report a time course of changes in plasma MDA levels and thus it is unknown whether an elevation is seen after as little as one day of exposure which did not occur in our study. Interestingly, a recent study that also used airway occlusions in anaesthetised rats to replicate an acute exposure to IH as occurs in OSA reported

an increase in plasma protein oxidation but did not demonstrate a noticeable change in plasma MDA levels (Celec 2012). Furthermore, IH may not be a stimulus capable of increasing levels of nitrotyrosine as two studies exposing rats to chronic IH were unable to show an effect (Philippi et al., 2010, Jun et al., 2008). It is therefore possible that in our study an alternative choice of biomarkers of oxidative stress from that of nitrotyrosine and MDA may have demonstrated an elevation in oxidative stress.

Respiratory plasticity

In contrast to our hypothesis that airway occlusions would elicit ventilatory LTF, ventilation during recovery room air breathing following six hours of airway occlusions with or without hyperglycaemia was not greater than during baseline room air breathing. Although the pattern of breathing in recovery from airway occlusions was stable, without any notable pauses that might indicate PaCO_2 had fallen below the apnoeic threshold, it is possible that a sizeable reduction in PaCO_2 as a result of post airway occlusion hyperventilation may still have inhibited the expression of respiratory LTF. Previous studies investigating respiratory LTF in anaesthetised animals following exposure to intermittent reductions in PI_{O_2} have considered that reduced chemoreceptor feedback as a result of hyperventilation-induced hypocapnia could restrain the expression of respiratory LTF. Therefore, these studies have often used supplemental CO_2 to ensure PaCO_2 does not fall below baseline levels during exposures to IH (Bach and Mitchell, 1996, Kinkead

and Mitchell, 1999, Baker and Mitchell, 2000). Indeed, in awake rats an increase in ventilation of only 20% was evident 60 minutes post acute IH but increased to 57% when supplemental CO₂ was subsequently given to return PaCO₂ to baseline levels (Olson et al., 2001). A major limitation of our study is that blood gases were only measured in four rats, none of which were exposed to airway occlusions. We can only speculate that the substantial hyperventilation following each airway occlusion may have lowered PaCO₂ below baseline levels. It is important to note that the average baseline PaO₂ of 74.3 ± 3.2 in the four rats assessed for blood gases is mildly hypoxic which could have been due to rats lying on their back partly restricting inspiration or as a consequence of anaesthesia. Therefore, the relatively high baseline PaCO₂ of 45.5 ± 1.5 may also have been elevated above normal normal levels. If animals in the airway occlusion group demonstrated similar baseline PaCO₂ and PaO₂ as would be expected then it is possible that airway occlusion induced hyperventilation could have caused a considerable lowering in PaCO₂ restraining the expression of ventilatory LTF without PaCO₂ falling below the apnoeic threshold.

Ventilation during exposure to 10% inspired O₂ during recovery from six hours of airway occlusions was not greater than during hypoxic exposure at baseline. This indicates the absence of any progressive augmentation of the HVR but similar to ventilatory LTF, it may have been restrained by lower PaCO₂ values. It is possible to assess PaCO₂ regularly during recovery to allow supplemental CO₂ to be used in

order to maintain PaCO_2 at baseline levels. However, this artificial control of PaCO_2 does not occur in OSA during sleep and thus by allowing PaCO_2 to change as it would naturally occur we have presented evidence to suggest ventilatory LTF and progressive augmentation of the HVR are unlikely to be expressed in OSA patients. However, as previously suggested by (Tadjalli et al., 2010) who was also unable to demonstrate ventilatory LTF following airway occlusions in anaesthetised rodents, it is possible that ventilatory LTF and progressive augmentation of the HVR are suppressed by different types and levels of anaesthesia.

An unexpected finding in this study was that in the HGAU group, ventilation in room air recovery was greater than room air baseline. As MAP pressure was not elevated above baseline levels it would seem unlikely that the greater ventilation was a result of a decreased level of anaesthesia. It is important to note that baseline measurements were made in fasted rats prior to the glucose infusion, whereas recovery measurements were made during continued glucose infusions. It is therefore possible an increased metabolic rate during recovery in response to the sustained hyperglycaemia could have increased PaCO_2 from baseline levels and thus mediated the elevation in ventilation. It is interesting to note that PaCO_2 in the three animals assessed for blood gases in the LGAU group demonstrated an average decrease in PaCO_2 of 4 ± 0.58 mmHg from baseline to recovery where the one rat in the HGAU group increased by 1 mmHg despite an increase in PaO_2 of 8 mmHg. Future research with blood gas measurements made in considerably more

animals is required before the responsible mechanisms of ventilatory LTF in the HGAU group and absence in the two airway occlusions groups can be established.

Summary

In contrast to our hypothesis, levels of plasma MDA and nitrotyrosine were not greater in animals exposed to simultaneous hyperglycaemia and repetitive airway occlusions. Indeed, neither simultaneous nor independent exposure to these stimuli over six hours was capable of increasing oxidative stress in healthy rats. Future studies aimed to investigate our hypothesis should use exposures lasting several weeks which have previously been shown to augment oxidative stress in animals and better resembles the chronic conditions of T2DM and OSA. Finally, in contrast to our hypothesis, six hours of repetitive airway occlusions with or without simultaneous hyperglycaemia did not evoke ventilatory LTF or progressive augmentation of the HVR.

7 CHAPTER 7 - SIMULTANEOUS EXPOSURE TO ACUTE INTERMITTENT HYPOXIA AND COMBINED POSTPRANDIAL HYPERGLYCAEMIA AND HYPERLIPIDAEMIA IN HEALTHY HUMANS

7.1 Introduction

IH is known to be a stimulus capable of inducing oxidative stress in humans and it is considered to be one of the primary candidates for mediating the development and progression of cardiovascular disease in OSA patients (Lavie and Lavie, 2009, Pack and Gislason, 2009, Garvey et al., 2009). Indeed, OSA patients demonstrate elevated levels of oxidative stress that are correlated to indices of hypoxic exposure such as the average nadir in S_aO_2 and the frequency of O_2 de-saturation events (Christou et al., 2009, Yamauchi et al., 2005). Furthermore, animals exposed to chronic IH express elevated levels of oxidative stress and develop cardiovascular disease, both of which are attenuated by antioxidant treatment (Burckhardt et al., 2008, Troncoso Brindeiro et al., 2007, Peng et al., 2006). In humans, exposure to acute IH also evokes increased oxidative stress (Pialoux et al., 2009, Lee et al., 2009).

Postprandial hyperlipidaemia (Ceriello et al., 2002) and postprandial hyperglycaemia (Ceriello et al., 2004) are also stimuli capable of inducing oxidative

stress in humans. It is likely that OSA patients who consume meals late in the evening will experience IH simultaneously with postprandial hyperglycaemia and hyperlipidaemia. This is even more likely in the 50 percent of T2DM patients who have coexisting OSA (Einhorn et al., 2007) because exaggerated and prolonged postprandial hyperglycaemia and hyperlipidaemia are hallmarks of T2DM. Based on a substantial body of evidence showing that simultaneous hyperglycaemia augments oxidative stress following IRI we hypothesised previously (chapter six) that simultaneous hyperglycaemia would elevate oxidative stress during IH. This was hypothesised because the mechanistic pathways responsible for the generation of ROS during IH and IRI may be similar because of the comparable pattern of hypoxia and re-oxygenation (Lavie, 2003, Pack and Gislason, 2009). In contrast to our hypothesis, we were unable to demonstrate a significant increase in oxidative stress in hyperglycaemic rats exposed to six hours of airway occlusions that induced IH mimicking what occurs in OSA. However, until now no study has investigated the effects of simultaneous exposure to IH and combined postprandial hyperglycaemia and hyperlipidaemia on oxidative stress in humans.

The high prevalence of T2DM in the OSA population is primarily due to the shared risk factor of excess weight (Einhorn et al., 2007) but recent evidence also suggests there is an independent association (Punjabi et al., 2004, Sulit et al., 2006, Reichmuth et al., 2005). It is well known that the diabetes health complication, *diabetes autonomic neuropathy*, can cause OSA as ventilatory control is impaired (Bottini et al., 2003, Bottini et al., 2000) but evidence is

emerging to suggest that OSA-induced IH may also cause the development and/or progression of T2DM. Indeed, rats and humans exposed to acute IH demonstrate a marked reduction in insulin sensitivity (Iiyori et al., 2007, Louis and Punjabi, 2009). Although (Louis and Punjabi, 2009) presented strong evidence showing IH caused a significant impairment in glucose removal during glucose infusion, no study until now has investigated the effects of IH on postprandial hyperglycaemia as would be experienced in OSA patients with T2DM.

In addition to investigating the effects of acute IH exposure on oxidative stress levels and a possible augmentation in postprandial hyperglycaemia a further opportunity is presented to investigate IH-induced respiratory plasticity. Previously, in chapters 4 and 5 we demonstrated the manifestation of ventilatory LTF in awake humans following an acute bout of hypoxic airway occlusions or acute exposure to intermittent reductions in $P_{I_{O_2}}$, respectively. In these studies ventilatory LTF was only expressed when P_{ETCO_2} was elevated above normal levels as previously shown by (Harris et al., 2006). However, all published studies specifically undertaken to investigate ventilatory LTF in humans and those performed in chapter 4 and 5 of this thesis have exposed participants to a relatively brief period of IH (< 90 minutes) (Harris et al., 2006, Wadhwa et al., 2008, Lee et al., 2009, Gerst et al., 2011). We considered it possible that an extended duration of IH exposure (i.e. six hours) could result in a greater activation of the responsible mechanisms for the development of ventilatory LTF and thus ventilatory LTF may

be expressed without supplemental CO₂. Furthermore, progressive augmentation of the HVR, like ventilatory LTF, has only been reported in humans if PETCO₂ is elevated above normal levels (Harris et al., 2006). However, as is the case for ventilatory LTF, no study has investigated whether exposure to acute IH for a period longer than ninety minutes is capable of inducing progressive augmentation of the HVR.

Study aims

The primary aim of this study was to investigate the effects of simultaneous exposure to six hours of IH and combined postprandial hyperglycaemia and hyperlipidaemia on oxidative stress in awake humans. We hypothesised that oxidative stress would be greater following simultaneous exposure to IH and combined postprandial hyperglycaemia and hyperlipidaemia than following either stressor alone. Furthermore, we aimed to investigate the interaction between IH and postprandial hyperglycaemia and hypothesised that IH would augment postprandial hyperglycaemia. The secondary aim of this study was to investigate whether ventilatory LTF and progressive augmentation of the HVR would be expressed under poikilocapnic conditions following a longer six-hour exposure to acute IH.

7.2 Methods

Ethical approval

After receiving detailed information on the procedures and risks all participants gave written consent to take part. The study was performed according to the latest revision of the Declaration of Helsinki and was approved by the local ethics committee (University of Birmingham ethical review committee).

Participants

We attempted to recruit overweight, middle aged, sedentary (< one hour per week of structured exercise) but otherwise healthy males. This population was chosen in order to resemble as closely as possible the characteristics of OSA patients with coexisting T2DM. In total, eleven healthy male individuals participated in the study. All participants were non-smokers, had no history of cardiovascular, respiratory and metabolic disease and were not taking any medication.

Protocol

All participants visited the laboratory for three experimental trials in a randomized order separated by at least 48 hours: *Trial 1* - IH following a high glucose/high lipid meal (IH + HG/HL), *Trial 2* - room air breathing following a high glucose/lipid meal (RA + HG/HL); and *Trial 3* - IH following a low glucose/low lipid meal (IH + LG/LL). Prior to experimental visits participants also undertook a preliminary visit to

become familiarised with the instrumentation and exposure to IH. For all experimental trials participants were asked to refrain from alcohol consumption and moderate to vigorous exercise for 24 hours and caffeine intake for 12 hours prior to arriving at the laboratory. All participants were supplied with three sets of identical mixed meals and they were instructed to consume the meal by 8 pm the evening before each experimental visit. Following consumption of the supplied evening meal participants were instructed to refrain from consuming additional food and to only drink water before visiting the laboratory the next morning at 7 am.

Preliminary visit

Participants were instrumented as previously described in chapter 5 (see: *Preliminary visit*). After breathing room air for approximately ten minutes they were exposed to a further ten minutes of IH in an identical way to what they would experience in the experimental visits as described in the next section.

IH protocol

We previously described a new model of airway occlusions that more realistically evokes IH as occurs in OSA than exposing individuals to intermittent reductions in PI_{O_2} (chapter 2). However, during the development of this model participants reported that it was too uncomfortable and tiresome to perform airway occlusions for longer than one hour. Therefore, in order to expose participants to IH of a similar duration to that experienced nightly by OSA patients (six hours) we chose

not to use that model. Therefore, participants were exposed to a continuous cycle of two minutes of hypoxia ($P_{ET_{O_2}} = 50$ mmHg) followed by two minutes of euoxia ($P_{ET_{O_2}} = 100$ mmHg) previously shown to elicit an elevation in oxidative stress in healthy humans (Pilaoux 2008). During hypoxia $P_{ET_{O_2}}$ was rapidly reduced to 50 mmHg and then it was tightly controlled at this value. During euoxia $P_{ET_{O_2}}$ was quickly brought back to 100 mmHg by inspiration of a single breath of 100 % O_2 and then maintained at this value. Supplemental CO_2 was not given and thus the IH exposure was poikilocapnic.

Experimental trials

In all experimental trials participants arrived at the laboratory at 7 am and had a cannula inserted into a vein in the antecubital or forearm region (Venflon, Becton Dickinson). They then positioned themselves comfortably on a couch in the supine position and were instrumented the same way as they were for the preliminary visit. Participants then breathed room air for fifteen minutes to allow measurements of resting ventilation to be assessed. Upon completion of baseline ventilatory measurements a fasting blood sample was drawn.

Participants then consumed a meal that was specific to the experimental trial being undertaken. In *Trial 1* (IH + HG/HL) and *Trial 2* (RA + HG/HL) participants consumed a croissant (45 g) with butter (15 g) and a dairy milkshake (skimmed milk 250 ml, double cream 100 ml and glucose 60 g). This meal provided 1110

kcal, 15 g protein, 75 g of lipid and 93 g carbohydrate of which 77 g were sugars. A dairy milkshake was chosen as it enabled ingestion of a large glucose load with the aim of eliciting a substantial hyperglycaemic spike. The buttered croissant was chosen to increase the total ingestion of lipid aimed at evoking hyperlipidaemia and thus enabled the required volume of cream in the milkshake to be limited ensuring it was palatable. In *Trial 3* (IH + LG/LL) participants consumed a bowl of high fibre cereal (37.5 g) with skimmed milk (100 ml). This meal provided 171 kcal, 8 g protein, 1 g of lipid and 31 g carbohydrate of which only 7 g were sugars and thus was chosen with the aim of not eliciting postprandial hyperglycaemia or hyperlipidaemia but preventing excess hunger in the fasted participants.

Participants were asked to consume their meal within five minutes. They were then asked to re-position themselves comfortably on the couch. Participants were then exposed to six hours of IH in *Trial 1* (IH + HG/HL) and *Trial 3* (IH + LG/LL) as described above (IH protocol). In contrast, participants breathed room air for six hours in *Trial 2* (RA + HG/HL). During *Trial 1* (IH + HG/HL) and *Trial 2* (RA+HG/HL) in an attempt to prolong postprandial hyperglycaemia to better mimic what occurs in T2DM patients, participants briefly removed their facemask and drank a high glucose drink (60 g glucose and 350 ml water) one hour after consumption of the meal. In *Trial 3* (IH+LG/LL) participants drank an equal volume of water (350 ml).

In all experimental trials in addition to a fasting blood sample, blood was drawn at 0.5, 1, 1.5, 2, 3 and 6 hours. Figure 7.1 is an illustration showing the different experimental protocols in the three trials.

Blood processing

Blood drawn at each collection time point was transferred from a 10 ml syringe into a 10 ml K3-EDTA vacutainer (Becton Dickinson) previously kept on ice. Blood was immediately centrifuged at 2000g at 4°C for 15 minutes. Plasma was then aspirated into several aliquots that were pre-cooled on ice and then immediately moved to storage at –80 °C.

Analytical procedures

Plasma samples were analysed for two different biological markers of oxidative stress (protein carbonylation and lipid peroxidation - LPO) and antioxidant capacity using three separate assays.

Protein carbonylation: The level of protein carbonylation was expressed relative to protein concentration and thus total plasma protein concentration was first determined using the bicinchoninic acid method (BCA) as previously described in chapter 6. The level of plasma protein carbonylation was assessed by an ELISA previously described by (Buss et al., 1997). Based on the BCA data plasma samples were diluted to 0.05 mg/ml in a coating buffer (50 mM Sodium Carbonate pH = 7.4). (1) Samples and standards (50 µl) were then added to a 96 well multi-

sorb plate (Nunc, Fisher Thermo Scientific, UK) and allowed to bind for one hour at 37 °C. (2) 50 µl of 2, 4-dinitrophenylhydrazine (1 mM) in 2 M hydrochloric acid was added to each well and incubated for one hour at room temperature. (3) Plates were blocked with 200 µl tris-buffered saline (TBS - Tween 20, 0.1 %) overnight at 4 °C. (4) 50 µl diluted (1:1000) mouse anti-DNP antibody was added to each well and incubated for two hours at 37 °C. (5) 50 µl diluted (1:5000) peroxidase labelled rat anti-mouse antibody was added to each well and incubated for one hour at 37 °C. (6) 50 µl citrate-phosphate based substrate (0.15 M, pH 5) was added to each well and incubated in the dark for 15/30 minutes. (7) The reaction was stopped with 2 M sulphuric acid and plates were read at 490 nm (Multiscan MS, Labsystems, Finland). Protein carbonylation values were expressed as nmol/mg of protein. Plates were washed four times between each step (1-7) with TBS (Tween 20, 0.5 %).

Lipid peroxidation: To determine the level of LPO in the plasma a spectrophotometric assay was used (Turner et al., 2011). 10 µl of samples, positive control (1:1000 hydrogen peroxide) and blank standard (distilled water) were added to 96 well plates in triplicate. 100 µl of reagent mix (0.2 M potassium phosphate (pH 6.2), 0.12 M potassium iodide, 0.15 mM sodium azide, 2 g/l Triton X, 0.1 g/l alkylbenzyltrimethylammonium chloride, 10 µM ammonium molybdate) was added to each well and incubated for 30 minutes at room temperature in the dark. Plates were read at 340 nm (Multiscan MS, Labsystems, Finland) and LPO

was determined using the Beer-Lambert Law (extinction coefficient of 24600). Plasma LPO values were expressed as nmol/ml plasma.

Antioxidant capacity: The antioxidant capacity of plasma was assessed using the FRAP assay as previously described in chapter 6 and expressed as μM of antioxidant power relative to ascorbic acid.

Assessment of plasma glucose and TAG

Plasma glucose and triglyceride (TAG) concentration was analysed using an ILAB automated analyser (Instrumentation Laboratory, Cheshire, UK).

Ventilatory analysis

In all experimental trials, \dot{V}_E , P_{ETCO_2} , P_{ETO_2} and S_aO_2 were recorded continuously. Ventilatory measurements were averaged during the last five minutes of room air breathing during the baseline and recovery periods. Furthermore, ventilatory measurements during the final minute of the first ten hypoxic episodes were averaged and compared to the average of the last ten hypoxic episodes in *Trial 1* (IH + HG/HL) and *Trial 3* (IH+LG/LL).

Statistical analysis

A two-way ANOVA with repeated measures in conjunction with a *post hoc* LSD test was used to determine whether there was a significant trial-by-time interaction for

each marker of oxidative stress (protein carbonylation, LPO), antioxidant capacity and ventilatory measurements (\dot{V}_E , P_{ETCO_2} , P_{ETO_2} and S_aO_2) and blood glucose and TAG. A one-way ANOVA with repeated measures was used to more closely determine whether there was a significant change over time in any of the above variables for each trial. Plasma glucose and TAG levels were also assessed by calculating the incremental area under the curve (*iAUC*) using the trapezoidal rule. *iAUC* responses represent the AUC with baseline reset to the fasting value and thus describe the change in concentration of plasma glucose and TAG from baseline (i.e. after meal consumption). A one-way ANOVA with repeated measures in conjunction with a *post hoc* LSD test was used to determine whether there was a difference in *iAUC* for plasma glucose and TAG between trials. The relationship between the percentage change from baseline in FRAP and LPO in each trial was examined using Pearson's product moment correlation coefficient. Values are expressed as mean \pm standard error and differences were considered significant if $P \leq 0.05$.

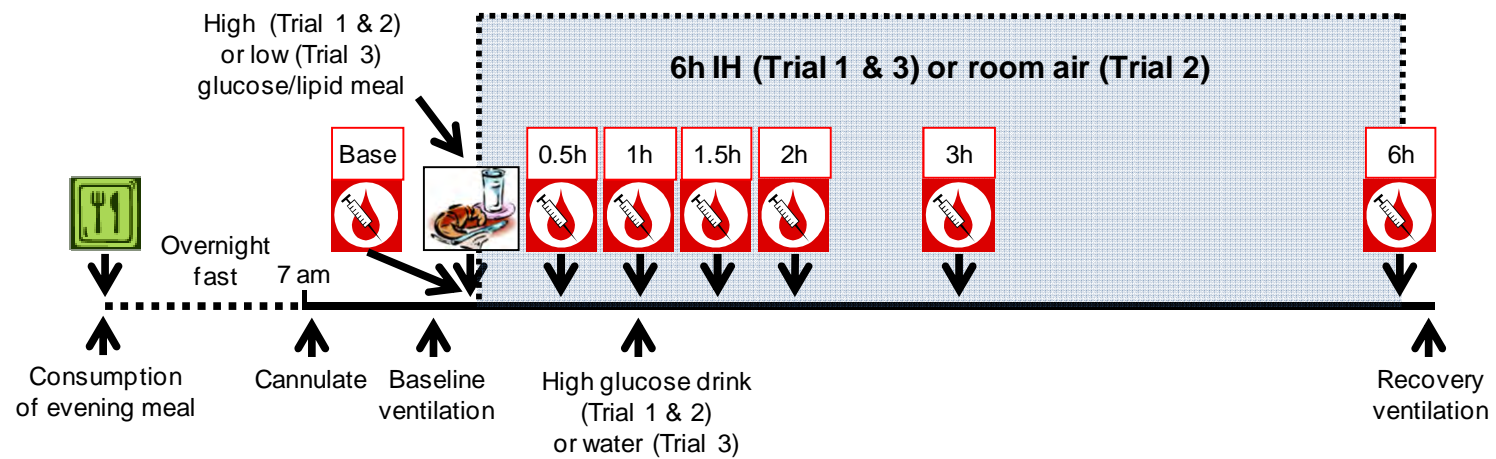


Figure 7.1: Schematic illustrating the protocol used for experimental trials. Blue square indicates six hour exposure to IH (Trial 1 and 2) or room air breathing (Trial 3)

7.3 Results

Participants

We found recruiting volunteers who closely fitted the inclusion criteria challenging due to the long laboratory visits required. Seven participants completed the entire study but four participants stopped short of completing one or more trials. Therefore, we were only able to make oxidative stress and ventilatory measurements for the seven participants who completed the full duration of each experimental trial. However, as a three-hour blood withdrawal was taken in all participants, baseline and postprandial blood glucose and TAG data is presented for eleven participants.

The seven healthy male participants who completed all three full experimental protocols and thus provided oxidative stress data had an average age of 44.9 ± 8.3 years, weight of 84.0 ± 14.6 kg, height of 174.8 ± 4.7 cm and a BMI of 27.4 ± 4.11 and where all sedentary. With the addition of the four participants who did not complete one or more experimental trials beyond the three hour blood sample the 11 participants had an average age of 44.5 ± 12.38 years, weight of 90.2 ± 20.1 kg, height of 175.6 ± 7.9 cm and a BMI of 29.0 ± 4.3 and where all sedentary. All values are mean \pm standard deviation.

Oxidative stress

Plasma LPO: Figure 7.2 shows plasma LPO levels at baseline and recovery in each of the different experimental trials. In *Trial 1* (IH + HG/HL) six of the seven participants demonstrated an increase in LPO levels and one did not change from baseline to recovery following simultaneous exposure to IH and combined postprandial hyperglycaemia and hyperlipidaemia. Analysis showed a significant increase over time (6.78 ± 0.66 vs. 8.34 ± 0.64 nmol/ml, $P = 0.006$). Furthermore, the change in LPO levels over time was significantly different ($P = 0.007$) from *Trial 2* (RA + HG/HL) and *Trial 3* (IH + LG/LL) where exposure to combined postprandial hyperglycaemia and hyperlipidaemia or IH independently did not cause an increase in LPO levels.

Plasma protein carbonylation: Figure 7.3 shows the level of plasma protein carbonylation at baseline and recovery in each of the different experimental trials. The level of protein carbonylation was not different between baseline and recovery in any of the experimental trials.

Antioxidant capacity: Figure 7.4 shows the antioxidant capacity of plasma at baseline and recovery in each of the different experimental trials. In *Trial 1* (IH + HG/HL) all seven participants demonstrated an increase in antioxidant capacity from baseline to recovery following simultaneous exposure to IH and combined postprandial hyperglycaemia and hyperlipidaemia. Analysis showed a significant

increase over time (835 ± 47.7 vs. 954 ± 46.3 μM , $P = 0.014$). In contrast, the change over time in *Trial 2* (RA + HG/HL) and *Trial 3* (IH + LG/LL) was variable and did not reach significance in either trial (*Trial 2*: 843 ± 44.3 vs. 873 ± 53.0 μM , $P = 0.490$; *Trial 3*: 831 ± 39.5 vs. 858 ± 44.1 μM , $P = 0.170$). The change in antioxidant capacity over time was however not significantly different between trials ($P = 0.148$).

Correlation between the change in antioxidant capacity and LPO levels

Figure 7.5 shows the correlation between the percentage change in antioxidant capacity of the plasma and LPO levels in all three experimental trials. A significant ($P = 0.004$) correlation of $r = 0.888$ was calculated for *Trial 1* (IH + HG/HL), a non significant ($P = 0.094$) correlation of $r = 0.56$ for *Trial 2* (RA + HG/HL) and a significant ($P = 0.030$) correlation of $r = 0.703$ for *Trial 3* (IH + LG/LL).

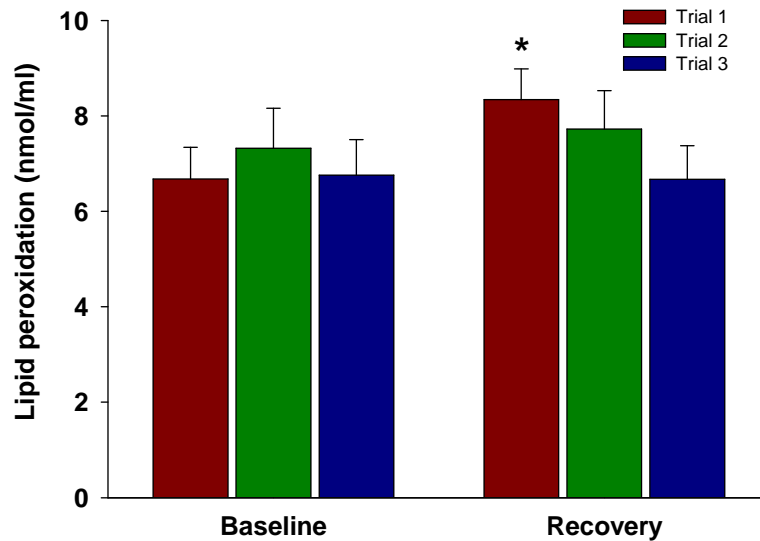


Figure 7.2: Levels of plasma lipid peroxidation (LPO) at baseline and recovery in all trials. LPO increased significantly over time in *Trial 1* (IH + HG/HL) ($P = 0.006$) but did not change in *Trial 2* (RA + HG/HL) or *Trial 3* (IH + LG/LL). * significantly different from baseline.

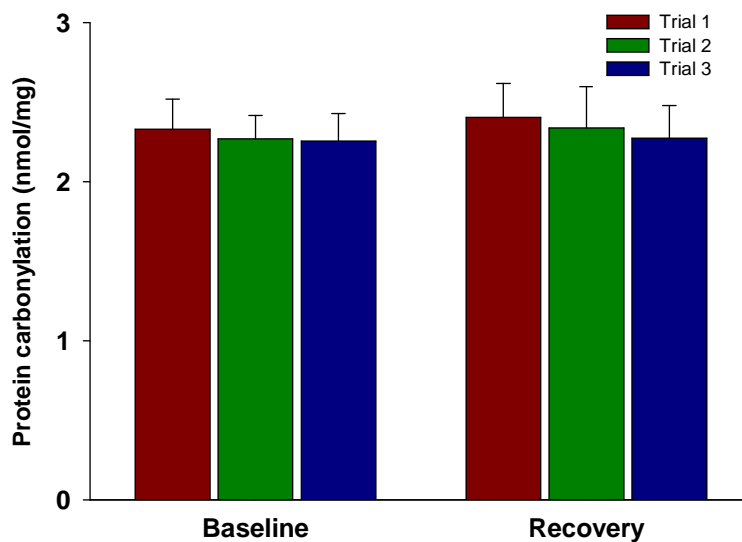


Figure 7.3: Levels of plasma protein carbonylation at baseline and recovery in all trials. There was no change from baseline to recovery in any of the trials.

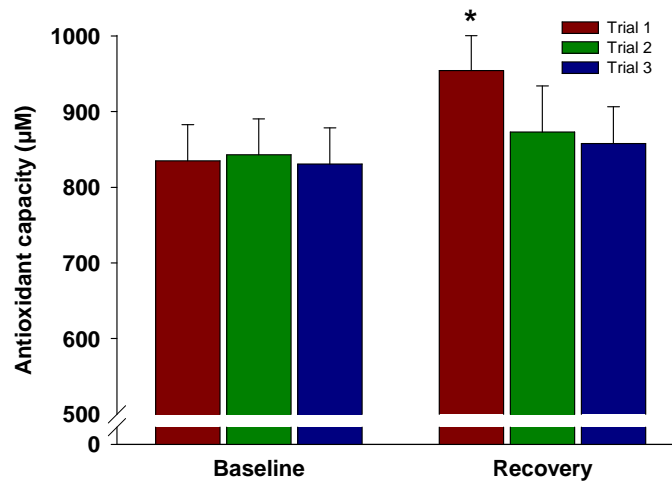


Figure 7.4: Plasma antioxidant capacity at baseline and recovery in all trials. Antioxidant capacity increased significantly over time in *Trial 1* (IH + HG/HL) ($P = 0.014$) but did not change in *Trial 2* (RA + HG/HL) or *Trial 3* (IH + LG/LL). * significantly different from baseline.

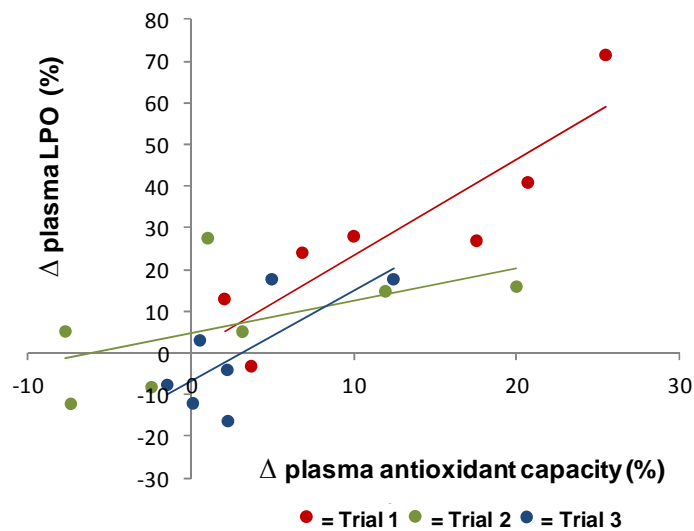


Figure 7.5: Graph showing the correlation between the percentage change from baseline of plasma lipid peroxidation (LPO) and antioxidant capacity in all trials. Pearson's correlation coefficient $r = 0.89$ for *Trial 1* (IH + HG/HL), $P = 0.004$, $r = 0.56$ for *Trial 2* (RA + HG/HL), $P = 0.094$, and $r = 0.73$ for *Trial 3* (IH + LG/LL), $P = 0.030$.

Respiratory Plasticity

Room air breathing: Table 7.1 shows average values for \dot{V}_E , P_{ETCO_2} , P_{ETO_2} and S_aO_2 during room air breathing at baseline and recovery in all experimental trials. Ventilatory LTF did not manifest in any of the experimental trials as \dot{V}_E was not different between baseline and recovery. There were no significant changes over time for any of the other variables (P_{ETCO_2} , P_{ETO_2} and S_aO_2) in any of the experimental trials.

Hypoxic exposures: Table 7.2 shows average values for \dot{V}_E , P_{ETCO_2} , P_{ETO_2} and S_aO_2 for the first ten hypoxic episodes and the last ten hypoxic episodes in *Trial 1* (IH + HG/HL) and *Trial 3* (IH + LG/LL). Progressive augmentation of the HVR was not evident during six hours of IH in *Trial 1* (IH + HG/HL) or *Trial 3* (IH + LG/LL) as \dot{V}_E during the last ten hypoxic episodes was not significantly different to the first ten in either trial. In *Trial 3* (IH + LG/LL) P_{ETCO_2} significantly decreased from baseline to recovery (35.9 ± 0.89 vs. 33.7 ± 1.27 mmHg, $P = 0.023$) but this change was not statistically different from *Trial 1* (IH + HG/HL). There were no significant changes between the first ten hypoxic episodes and the last ten hypoxic episodes for P_{ETO_2} or S_aO_2 in either of the experimental trials.

Postprandial hyperglycaemia

Figure 7.6 shows the change in plasma glucose levels over time in each of the different experimental trials. As was expected, plasma glucose levels in *Trial 3* (IH + LG/LL) demonstrated a small increase following the low glucose meal, peaking at 30 minutes and then gradually declining thereafter, falling slightly below baseline at three hours ($P = 0.001$). Plasma glucose levels also changed over time following the high glucose meal and drink in *Trial 1* (IH + HG/HL) ($P = 0.001$) and *Trial 2* (RA + HG/HL) ($P = 0.050$) but despite identical glucose loads the change over time in these studies was significantly different ($P = 0.020$). Plasma glucose levels were similar in *Trial 1* (IH + HG/HL) and *Trial 2* (RA + HG/HL) at 30 minutes when they peaked in both experimental trials in response to the high glucose meal. However, plasma glucose levels then remained above baseline levels at sixty minutes in *Trial 1* (IH + HG/HL) whereas they decreased close to baseline levels in *Trial 2* (RA + HG/HL). Furthermore, consumption of the high glucose drink one hour after consuming the meal caused a secondary spike in glucose levels at 90 minutes in both trials but this was significantly greater in *Trial 1* (IH + HG/HL) than *Trial 2* (RA + HG/HL).

Figure 7.7 shows plasma glucose *iAUC* for all trials. Analysis showed plasma glucose levels were significantly different between trials ($P = 0.002$). *Post hoc* analysis showed plasma glucose *iAUC* was significantly greater in *Trial 1* (IH + HG/HL) than both *Trial 2* (RA + HG/HL) and *Trial 3* (IH + LG/LL) but that the

difference between *Trial 2* (RA + HG/HL) and *Trial 3* (IH + LG/LL) was not statistically significant.

Postprandial hyperlipidaemia

Figure 7.8 shows how plasma TAG levels changed over time in each of the different experimental trials. Plasma TAG gradually increased over time in *Trial 1* (IH + HG/HL) ($P = 0.004$) and *Trial 2* (RA + HG/HL) ($P = 0.000$) following consumption of a high lipid meal and did not change following consumption of the low lipid meal in *Trial 3* (IH + LG/LL). Figure 7.9 shows plasma TAG *iAUC* for all trials. Analysis showed plasma TAG levels were significantly different between trials ($P = 0.001$). *Post hoc* analysis showed plasma TAG *iAUC* was significantly greater in *Trial 1* (IH + HG/HL) and *Trial 2* (RA + HG/HL) from *Trial 3* (IH + LG/LL) as was expected with the different lipid loads.

Time	Trial 1 - (IH+HG/HL)		Trial 2 - (RA+HG/HL)		Trial 3 - (IH+LG/LL)	
	Baseline	Recovery	Baseline	Recovery	Baseline	Recovery
VE (l/min)	8.9 ± 0.4	9.4 ± 0.4	9.0 ± 0.4	9.1 ± 0.3	9.2 ± 0.5	8.6 ± 0.3
P _{ET} CO ₂ (mmHg)	39.5 ± 1.3	36.8 ± 0.7	37.9 ± 1.0	38.5 ± 0.5	38.0 ± 1.0	37.1 ± 1.1
P _{ET} O ₂ (mmHg)	100 ± 2.7	99 ± 1.2	103 ± 2.4	101 ± 0.9	103 ± 2.0	99 ± 1.1
S _a O ₂ (%)	96.7 ± 0.3	96.7 ± 0.2	96.8 ± 0.4	97.3 ± 0.2	96.6 ± 0.4	96.5 ± 0.2

Table 7.1: Averaged ventilatory measurements during baseline and recovery room air breathing in all trials.

Time	Trial 1 - (IH+HG/HL)		Trial 3 - (IH+LG/LL)	
	Early Hypoxia	Late Hypoxia	Early Hypoxia	Late Hypoxia
VE (l/min)	12.7 ± 0.6	13.7 ± 0.7	12.4 ± 0.9	12.6 ± 0.8
P _{ET} CO ₂ (mmHg)	35.6 ± 1.0	34.0 ± 0.7	35.9 ± 0.9	33.7 ± 1.3 *
P _{ET} O ₂ (mmHg)	49.5 ± 0.6	48.9 ± 1.0	50.4 ± 1.4	49.0 ± 0.8
S _a O ₂ (%)	85.9 ± 0.6	86.1 ± 0.7	87.2 ± 0.6	86.6 ± 0.5

Table 7.2: Averaged ventilatory measurements for the first ten hypoxic episodes (early hypoxia) and the last ten hypoxic episodes (late hypoxia) of a six-hour exposure to IH, in *Trial 1* (IH + HG/HL) and *Trial 3* (IH + LG/LL). * significantly different from early hypoxia of the same trial.

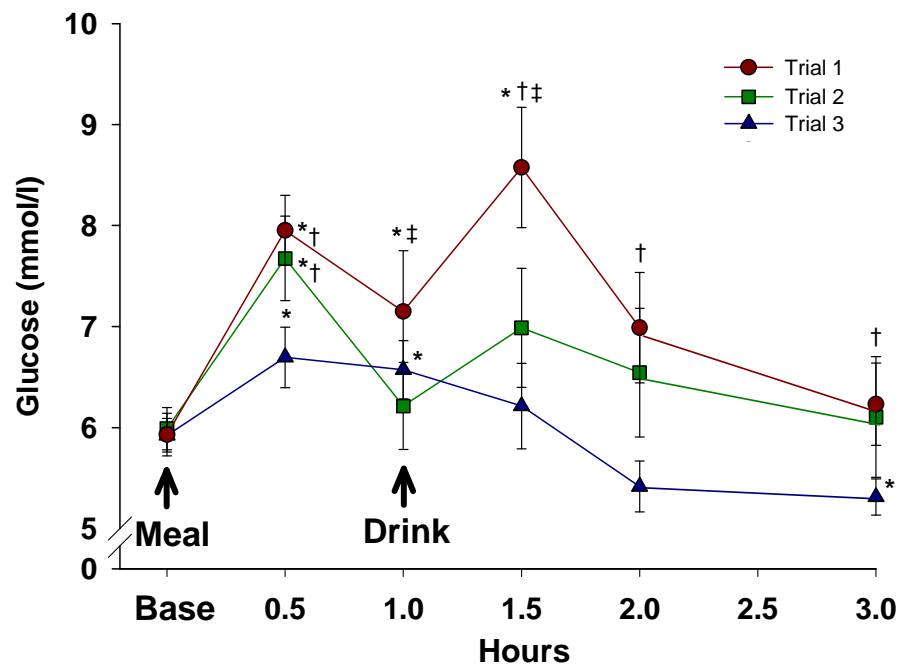


Figure 7.6: Plasma glucose levels in *Trial 1* (IH + HG/HL), *Trial 2* (RA + HG/HL) and *Trial 3* (IH + LG/LL) at baseline, and following a high (*Trial 1* and *Trial 2*) or low (*Trial 3*) glucose meal with additional high (*Trial 1* and *Trial 2*) or low glucose drink (*Trial 3*) one hour later. † significantly different from *Trial 3* (IH + LG/LL). ‡ significantly different *Trial 2* (RA + HG/HL).

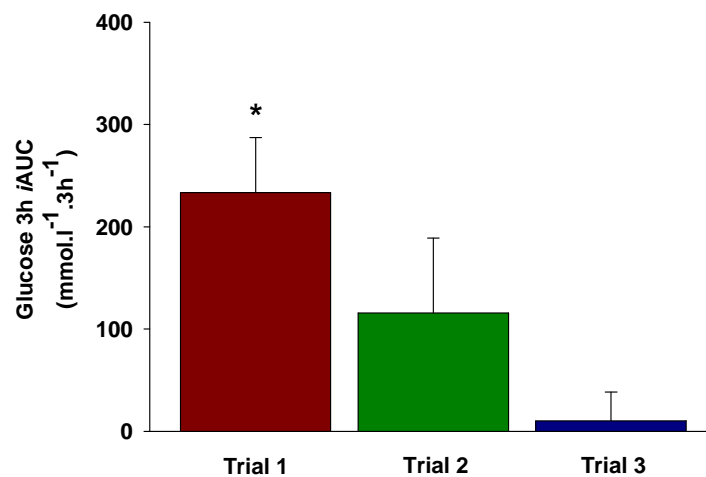


Figure 7.7: Plasma glucose three-hour incremental area under the curve (iAUC) for *Trial 1* (IH + HG/HL), *Trial 2* (RA + HG/HL) and *Trial 3* (IH + LG/LL). * significantly different from *Trial 2* (RA + HG/HL) and *Trial 3* (IH + LG/LL).

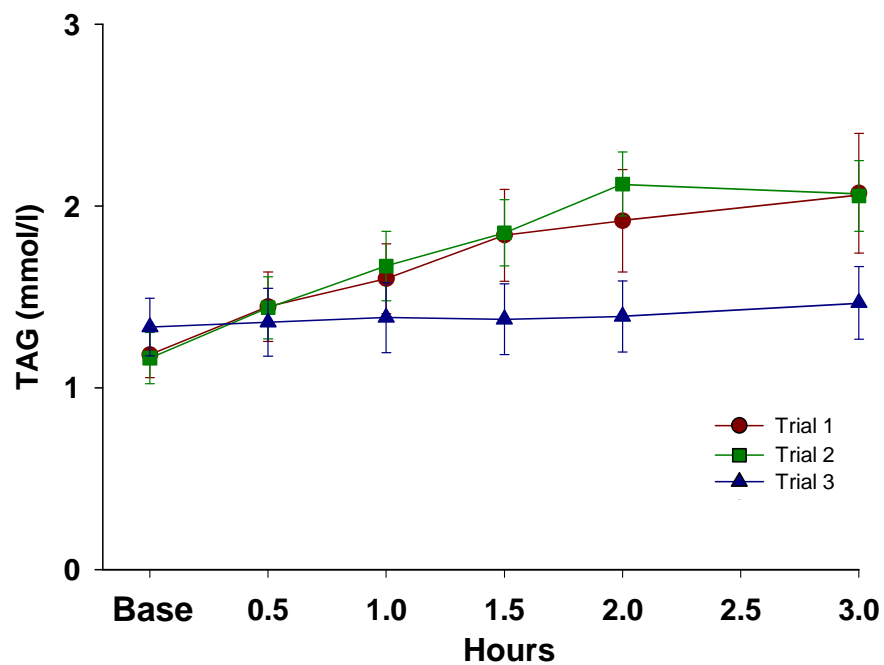


Figure 7.8: Plasma triglyceride (TAG) levels in *Trial 1* (IH + HG/HL), *Trial 2* (RA + HG/HL) and *Trial 3* (IH + LG/LL) at baseline, and following a high (*Trial 1* and *Trial 2*) or low (*Trial 3*) lipid meal.

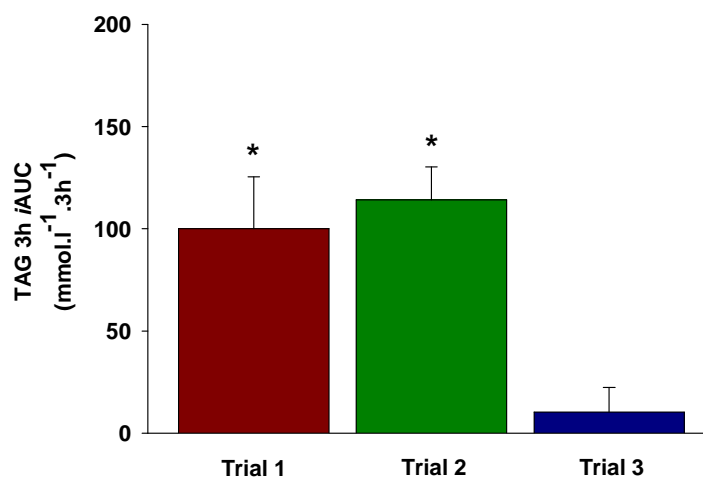


Figure 7.9: Plasma triglyceride (TAG) three-hour incremental area under the curve (iAUC) for *Trial 1* (IH + HG/HL), *Trial 2* (RA + HG/HL) and *Trial 3* (IH + LG/LL). * significantly different from *Trial 3* (IH + LG/LL).

7.4 Discussion

Primary findings

The primary findings of this study are four fold: 1) exposure to six hours of IH simultaneously with combined postprandial hyperglycaemia and hyperlipidaemia significantly increased oxidative stress (LPO), whereas independent exposures that did not; 2) the elevation in oxidative stress is closely correlated to an elevation in plasma antioxidant capacity; 3) exposure to IH augments postprandial hyperglycaemia and finally, 4) six hours exposure to IH with or without simultaneous combined postprandial hyperglycaemia and hyperlipidaemia does not elicit ventilatory LTF or progressive augmentation of the HVR.

Oxidative stress

LPO represents oxidative damage of lipids most commonly associated with polyunsaturated fatty acids that make up the major component of cell membranes (Niki, 2009). We used an assay previously shown to be sensitive to changes in plasma LPO levels in healthy humans (Turner et al., 2011) and were able to demonstrate a significant elevation in LPO following six hours of IH if participants also consumed a high glucose/lipid meal (*Trial 1*). In contrast to a previous report (Pialoux et al., 2009) six hours exposure to IH without consumption of a high glucose and lipid meal in our study did not increase LPO levels (*Trial 3*) and nor did consumption of the high glucose and lipid meal independently (*Trial 2*). It has been

demonstrated that LPO can alter cell membrane structure and lead to a loss of cellular function in addition to promoting a pro-atherogenic form of low-density lipoprotein (i.e. oxidised-LDL) (Niki, 2009, Yoshida and Kisugi, 2010). Indeed, numerous studies have shown elevated levels of LPO in plasma and tissues in patients with cardiovascular disease (Niki, 2009, Rumley et al., 2004). Therefore, our results may have implications for the development and/or progression of cardiovascular disease in OSA patients with T2DM consuming high glucose and lipid evening meals.

Numerous human diseases are associated with elevated levels of protein carbonylation such as diabetes, Alzheimer's disease and chronic lung and kidney disease (Dalle-Donne et al., 2003). Therefore, we thought this was an appropriate biological marker of oxidative stress to assess in this study. However, in contrast with LPO levels there was no elevation in plasma protein carbonylation in recovery in any of the three experimental trials. Proteins are susceptible to oxidation particularly from end products of LPO (e.g. hydroxynonenal (4-HNE) and malonaldehyde (MDA)) that react with proteins causing carbonyl groups to be added (Berlett and Stadtman, 1997). Although speculative it is possible that in this study the increase in LPO in healthy albeit overweight humans following acute exposure to IH and postprandial hyperglycaemia and hyperlipidaemia is insufficient in magnitude or not sustained for a required duration to cause a noticeable increase in plasma MDA levels. If this is correct it may explain the absence of any increase in plasma protein carbonylation. In chapter 6 we showed that in rats

exposure to sustained hyperglycaemia and IH, MDA levels were not increased but having not measured plasma LPO levels it is not possible to make further judgment on this.

Possible mechanisms for increased oxidative stress

To the best of our knowledge this is the first study performed in humans to investigate the effects of simultaneous exposure to IH and combined postprandial hyperglycaemia and hyperlipidaemia on oxidative stress. The study was not designed to specifically investigate potential mechanisms of how hyperglycaemia and/or hyperlipidaemia could augment oxidative stress during IH exposure. Rather, we aimed to describe whether there was an effect under conditions aimed to mimic a real life situation (i.e. a mixed meal with six hours IH to mimic that occurring during a night of OSA). Although future studies are required to investigate the possible mechanisms that are involved we have identified some possibilities.

Firstly, during acute periods of ROS overproduction the level of subsequent oxidative stress is greatly limited by the body's antioxidant defence system. It is possible that greater levels of ROS production during simultaneous exposure to IH and combined postprandial hyperglycaemia and hyperlipidaemia could lead to substantially greater levels of oxidative stress than during individual stressors due to the greater level of ROS overwhelming antioxidant defences.

Secondly, in healthy insulin-sensitive individuals, postprandial hyperglycaemia does not produce an elevation in plasma lipid oxidation even after ingestion of as much as 150 g of glucose (Fisher-Wellman and Bloomer, 2010). However, in T2DM patients who experience exaggerated postprandial hyperglycaemia there is significant elevation in lipid oxidation after much smaller glucose loads (Ceriello et al., 2004). It is possible that the increase in LPO in *Trial 1* (IH + HG/HL) is partly due to the IH-induced augmentation in postprandial hyperglycaemia.

Thirdly, hyperglycaemia may not have independently increased ROS production in the healthy albeit overweight participants in this study but could have augmented IH-induced generation of ROS. Studies have shown acute exposure to hyperglycaemia does not cause an elevation in oxidative stress in insulin sensitive humans and rodents (Fisher-Wellman and Bloomer, 2010, Kayali et al., 2003, Cakatay and Kayali, 2006). However, recent evidence from experimental animal studies has shown that acute hyperglycaemia augments the generation of ROS and oxidative stress during exposure to IRI (Hirose et al., 2008, Bemeur et al., 2007, Tsuruta et al., 2010). Indeed, in two of these studies acute hyperglycaemia *per se* had no effect on ROS generation or oxidative stress but greatly augmented the response during both ischaemia and early reperfusion (Hirose et al., 2008, Tsuruta et al., 2010). As previously discussed in detail (General introduction: *Mechanisms for IH-induced oxidative stress*) the mechanistic pathways responsible for the generation of ROS during IH and ischaemia-reperfusion injury may be similar because of the comparable pattern of hypoxia and re-oxygenation (Lavie,

2003, Pack and Gislason, 2009). Therefore it is tempting to speculate that simultaneous hyperglycaemia could also augment IH-induced ROS generation as it does for IRI.

Although speculative and requiring complex studies to confirm its existence we have developed a working model for how hyperglycaemia could increase ROS generation during IRI and IH exposure that involves the enzyme xanthine oxidase. A detailed description of the possible role that xanthine oxidase plays in mediating the elevation in ROS during IRI is given previously (General introduction: *Mechanisms for IH-induced oxidative stress*). In brief, xanthine oxidase catalyses the oxidation of hypoxanthine to xanthine and subsequently to uric acid with O₂ and produces superoxide. During ischaemia the non-ROS producing enzyme xanthine dehydrogenase is converted to xanthine oxidase (McKelvey et al., 1988, Engerson et al., 1987) and a reduction in oxidative phosphorylation results in an increase in the availability of hypoxanthine to be catalysed by xanthine oxidase (Thompson-Gorman and Zweier, 1990, Gimpel et al., 1995, Zweier et al., 1994).

Two lines of evidence have recently emerged that suggest that the hyperglycaemic-induced increase in ROS production during IRI and presumably IH could be mediated by hyperglycaemia up-regulating the activity of xanthine oxidase. Firstly, levels of allantoin, a marker of oxidative stress specific to xanthine oxidase activity (Serkova et al., 2005) assessed 24 hours after simultaneous exposure to hyperglycaemia and renal IRI, were significantly greater

than IRI alone or when hyperglycaemia occurred two hours after reperfusion (Hirose et al., 2008). Secondly, protein kinase C (PK-C) dependent activation of NAD(P)H oxidase during hyperglycaemia may increase ROS production (Brownlee, 2001) and it has been demonstrated that ROS production from NAD(P)H oxidase regulates xanthine oxidase activity (McNally et al., 2003). Therefore, during simultaneous hyperglycaemia and IRI and presumably IH, hyperglycaemia-induced activation of NAD(P)H oxidase may increase ROS production indirectly by increasing xanthine oxidase activity as illustrated below (Figure 7.10).

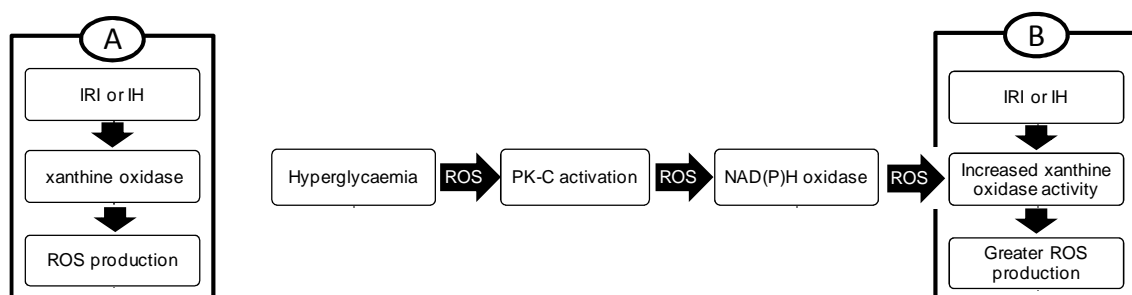


Figure 7.10: Schematic demonstrating how hyperglycaemia may increase xanthine oxidase generated ROS during ischaemia-reperfusion injury (IRI) or IH. (A) IRI or IH under euglycaemic conditions. (B) IRI or IH under hyperglycaemic conditions.

Most of the uric acid circulating in the blood is in the form urate, which is responsible for over half of the total antioxidant capacity of plasma in humans (Maxwell et al., 1997). Interestingly, uric acid is produced during activation of xanthine oxidase (General introduction: *Mechanisms for IH-induced oxidative stress*) and OSA patients express elevated levels of uric acid that are normalised

following CPAP treatment (Sahebji, 1998). Therefore, it might be expected that if hyperglycaemia does augment the increase in xanthine oxidase activity during IH there would be greater uric acid production leading to greater plasma antioxidant capacity. Indeed, endothelial cells that are in contact with the plasma and will be most exposed to hyperglycaemia and to fluctuations in arterial oxygen levels are known to have high concentrations of xanthine oxidase. We used a FRAP assay to assess total antioxidant capacity of the plasma reflected by the sum of all antioxidants (i.e. endogenous and exogenous) including uric acid (Bartosz, 2010). Interestingly, we demonstrated there was a significant increase in antioxidant capacity between baseline and recovery in *Trial 1* (IH + HG/HL) following six hours of exposure to simultaneous IH and combined postprandial hyperglycaemia and hyperlipidaemia. There was not a significant increase over time in either *Trial 2* (RA + HG/HL) or *Trial 3* (IH + LG/LL) although the change in antioxidant capacity was not statistically different between the trials. Furthermore, although correlations on such a small number of participants should be analysed with caution it was interesting to see such a strong correlation between the change from baseline in LPO with that of antioxidant capacity in *Trial 1* (IH + HG/HL).

Alternatively, the increase in antioxidant capacity may be a compensatory mechanism by the body in response to an increase in ROS generation that does not specifically indicate enhanced xanthine oxidase activity. Although chronic oxidative stress may result in a depletion of antioxidants as demonstrated in patients suffering from a number of diseases (Barcelo et al., 2006, Baynes, 1991)

mammals appear to be able to rapidly increase their antioxidant defence capacity during acute periods of mild to moderate oxidative stress (Guzel et al., 2000, Ji, 2008, Rauchova et al., 2005). Indeed, acute hypoxia increased antioxidant enzyme activity of catalase and SOD in erythrocytes in rats (Guzel et al., 2000, Rauchova et al., 2005). Redox-sensitive signalling has been shown to increase gene expression for enzymatic antioxidants within 15 minutes of exercise and thus may well occur during six hours IH exposure (Ji, 2008).

Postprandial hyperglycaemia and hyperlipidaemia

Postprandial hyperlipidaemia following the high lipid meal was not different during IH or room air conditions. In contrast, postprandial hyperglycaemia was augmented during IH as shown by a significantly greater three-hour *iAUC*.

Several epidemiological studies have demonstrated that OSA is associated with glucose intolerance, insulin resistance and T2DM and that this is independent of other comorbidities such as obesity (Punjabi et al., 2004, Sulit et al., 2006, Reichmuth et al., 2005). Although sleep fragmentation is likely to play a major role in mediating these changes in glucose control (Punjabi and Polotsky, 2005) acute IH of durations as little as nine hours induced insulin resistance in lean rodents (Iiyori et al., 2007) and just 30 minutes exposure has been shown to decrease insulin sensitivity in humans (Oltmanns et al., 2004). Only one study prior to ours has investigated the effects of acute IH on glucose metabolism in humans (Louis and Punjabi, 2009). (Louis and Punjabi, 2009) exposed participants to eight hours

of acute IH that was similar to that used in our study but used the frequently sampled intravenous glucose tolerance test (IVGTT) to investigate the effects of acute IH on insulin-dependent and insulin-independent mechanisms of blood glucose removal. They demonstrated that acute IH decreased insulin sensitivity without a compensatory increase in insulin secretion. This is in contrast to the normal physiological response to a reduction in insulin sensitivity where increased insulin secretion occurs in order to maintain blood glucose homeostasis (Kahn, 2003). The authors speculate this may have been due to acute IH also inhibiting pancreatic ability to compensate (Louis and Punjabi, 2009). Furthermore, they demonstrated acute IH caused a significant decrease in insulin-independent removal of blood glucose (i.e. glucose mediated disposal).

The authors of that study suggest a number of potential mechanisms that may be responsible for the acute IH-induced impairment in glucose metabolism. Firstly, acute IH is known to increase sympathetic activity in humans (Xie et al., 2001, Xie et al., 2000, Tamisier et al., 2005, Cutler et al., 2004b, Cutler et al., 2004a, Leuenberger et al., 2005) and studies have shown that adrenalin inhibits insulin secretion (Porte, 1967), augments hepatic glucose output by increased gluconeogenesis and glycogenolysis (Exton and Park, 1968, Sherline et al., 1972) and decreases hepatic and peripheral tissue insulin sensitivity (Deibert and DeFronzo, 1980). Secondly, IH may increase circulating levels of cortisol as previously shown during sustained hypoxia (Coste et al., 2005, Humpeler et al., 1980) which has been shown to increase hepatic glucose output, decrease insulin

secretion and induce insulin resistance (Andrews and Walker, 1999, Lambillotte et al., 1997). Thirdly, an intriguing mechanism responsible for altered glucose metabolism during acute IH is that ROS may play a role. It has been shown that ROS can activate cellular stress-sensitive pathways impairing cellular signaling leading to reduced cellular insulin sensitivity (Bloch-Damti and Bashan, 2005). Furthermore, ROS are known to impair pancreatic β -cell function that is a key mechanism for reduced insulin secretion (Robertson, 2006, Evans et al., 2003) and it was recently shown that exposure of rodents to IH caused increased β -cell death mediated by ROS (Xu et al., 2009). In addition to the mechanisms proposed by (Louis and Punjabi, 2009) it is possible that IH could have augmented postprandial hyperglycaemia by altering gastrointestinal blood flow allowing greater uptake of glucose and/or increasing absorption rates of glucose from the gut. Although speculative, it is possible that IH may cause a redistribution of blood flow away from organs capable of removing glucose from the blood such as skeletal muscle and the liver, possibly mediated by elevated sympathetic activity. Interestingly (Leuenberger et al., 2005) showed that 30 minutes exposure to IH increased forearm vascular resistance in healthy humans. Future studies investigating whether exposure to IH attenuates hepatic blood flow could utilise Doppler ultrasound to non-invasively determine portal vein blood flow.

As already mentioned the present study was not designed to specifically investigate the effects of IH on postprandial hyperglycaemia. There are a number of factors in the study design that limit our understanding and drawing mechanistic

conclusions should be done cautiously. Firstly, unlike an oral glucose tolerance test (OGTT) that is commonly used to assess glucose handling and that involves the ingestion of glucose only, our participants consumed a mixed meal that although was high in glucose it was also high in lipid and contained complex carbohydrates (i.e. starch rather than sugar). Although unlikely, it cannot be ruled out that IH alters the breakdown and ingestion of carbohydrate not already in a glucose form and it is not known whether the presence of high levels of ingested lipid also interacts in some way with IH to alter plasma glucose levels. Secondly, the temporal pattern of blood sampling in our study was relatively infrequent (every 30 minutes) compared to that commonly used in an OGTT (every 15 minutes). The inability to gain a clear picture of the postprandial plasma glucose profile because of the poor temporal resolution is worsened by the ingestion of the glucose drink at one hour that causes a secondary short-lived elevation in plasma blood glucose levels. Finally we did not measure plasma insulin levels in our study and thus cannot make any conclusions regarding what mediates the augmentation in postprandial hyperglycaemia.

Respiratory plasticity

All previous attempts to elicit respiratory LTF in awake humans under eucapnic or poikilocapnic conditions exposed individuals to a relatively brief period of acute IH (< 90 minutes) and were unsuccessful (Mateika and Sandhu, 2011). We considered it possible that a greater duration of acute IH could lead to a greater activation of the responsible mechanisms for respiratory LTF and thus ventilatory

LTF could be expressed. However, exposure to six hours of acute IH with or without simultaneous exposure to combined postprandial hyperglycaemia and hyperlipidaemia did not evoke ventilatory LTF in our participants. These findings add support to our previous findings (chapter 4 and 5) and to existing published research (Harris et al., 2006, Wadhwa et al., 2008, Lee et al., 2009, Gerst et al., 2011) that demonstrates the requirement for raising P_{ETCO_2} above normal levels for ventilatory LTF to be expressed.

In addition to raised P_{ETCO_2} being a requirement for the expression of ventilatory LTF, previous studies have demonstrated the manifestation of progressive augmentation of the HVR is also reliant on elevated P_{ETCO_2} (Harris et al., 2006, Mateika et al., 2004). We speculated that progressive augmentation of the HVR may be evident following acute poikilocapnic IH of a longer duration than previously used by studies (< 90 minutes). However we were unable to demonstrate any augmentation in ventilation during the final ten hypoxic episodes compared with that of the initial ten hypoxic episodes during six hours exposure to acute IH with or without combined postprandial hyperglycaemia and hyperlipidaemia.

Summary

In summary, we have demonstrated that simultaneous but not individual exposure to six hours of IH and combined postprandial hyperglycaemia and hyperlipidaemia

elicited a significant increase in oxidative stress. Furthermore, this increase in oxidative stress appears to be associated with an increase in plasma antioxidant capacity. These findings could have implications for OSA patients who consume high glucose/lipid meals prior to sleep, especially those with coexisting T2DM who experience augmented postprandial hyperglycaemia and hyperlipidaemia. However, the underlying mechanisms that are responsible for the augmentation in oxidative stress during simultaneous exposure to IH and combined postprandial hyperglycaemia and hyperlipidaemia remain to be determined. In addition, we have supplied evidence to suggest that postprandial hyperglycaemia may be augmented during simultaneous exposure to IH. This also has implications for OSA patients with coexisting T2DM. Finally, despite a lengthy duration of IH in our study compared with previous studies (six hours vs. < 90 minutes) ventilatory LTF and progressive augmentation of the HVR were not expressed. This finding provides support for the requirement of raising P_{ETCO_2} levels in the manifestation of IH-induced respiratory plasticity in awake humans.

8 CHAPTER 8 - GENERAL DISCUSSION

8.1 Development of an airway occlusion model of OSA

The first aim of this thesis was to develop an airway occlusion model of OSA that would expose humans to as many of the events occurring in OSA with or without IH. If successful, such a model would allow a more comprehensive investigation into the effects of OSA-related IH. We feel that the newly developed airway occlusion model of OSA as described in chapter 2 is superior to other existing human models of OSA for a number of reasons. Firstly, generation of negative intrathoracic pressures as is experienced by OSA patients was made possible with the inclusion of an automated valve in the breathing circuit. Furthermore, we feel the model was effective in replicating a number of other physiological events that occur during repetitive obstructive apnoeas in OSA (e.g. tachycardia, blood pressure rises, hypercapnia etc). Finally, the DEF system was an effective tool in isolating the effects of IH.

However, long exposures to airway occlusions were tiring and tedious and the value of using this model was severely limited. In retrospect, we do not believe that one hour of airway occlusions is sufficient time to induce measurable changes in oxidative stress and possibly other physiological consequences that occur in OSA.

Therefore, although we used this model in chapter 3 and 4 to investigate a number of hypothesis regarding the pulmonary circulation and respiratory plasticity, in later chapters we followed different approaches (an animal study and intermittent reductions in inspired PI_{O_2} in humans).

8.2 Investigating the effects of acute IH as it occurs in OSA on pulmonary arterial pressure

PAP is known to rise during each occluded apnoea in OSA patients but at present the responsible mechanism(s) are not fully understood. Because hypoxia is present in each apnoea it is widely considered that HPV is responsible for the gradual rise in PAP. In support for this hypothesis, inhibition of hypoxia during occluded apnoeas in dogs with prior O_2 supplementation completely abolished the rise in PAP (Schneider et al., 2000, Iwase et al., 1992). However, this has only been incompletely investigated in OSA patients (Marrone et al., 1992). Therefore, in chapter 3 we attempted to use our newly developed model of OSA to expose participants to airway occlusions with and without hypoxia in order to investigate whether HPV is responsible for the rise in PAP.

Previous animal and human studies investigating the effect of apnoeas on the pulmonary circulation have used Swan-Ganz catheters to measure PAP. Although Swan-Ganz catheters are considered to be the gold standard for assessing PAP,

the technique is highly invasive so its use in experimental research is limited. Therefore, we attempted to use an established Doppler ultrasound technique to measure systolic PAP (SPAP). This technique has been extensively used during continuous exposures to hypoxia but it has not been used during airway occlusions. Disappointingly, we were forced to conclude that Doppler ultrasound is not a suitable tool to assess changes in SPAP during airway occlusions because the accuracy of measurements were compromised by the brief duration of apnoeas and substantial rib cage movements. Consequently, we could not evaluate the contribution of HPV to the elevation in PAP during airway occlusions nor could we determine whether HPV increased in sensitivity over time.

Although there exists a substantial body of research investigating the time course of HPV in animals and humans during continuous exposure to hypoxia little is known about the time course to IH in animals and none at all in humans. However, evidence from one study in dogs showed that the sensitivity of HPV increases during exposure to IH. Although we were unable to use Doppler ultrasound to assess SPAP during airway occlusions, this technique has previously been used to assess HPV during longer hypoxic exposures (Talbot et al., 2005). Therefore, in chapter 4 participants were exposed to either one hour of hypoxic airway occlusions or one hour of unrestricted room air breathing followed by a 20-minute hypoxic exposure when SPAP was continuously measured. We hypothesised that HPV would be augmented following exposure to hypoxic airway occlusions. However, in contrast to our hypothesis the magnitude and pattern of HPV during

the 20-minute hypoxic exposure following one hour of hypoxic airway occlusions was not different to that following unrestricted room air breathing. Future studies are required to see if longer exposures to OSA related-IH are capable of sensitising HPV.

8.3 IH and oxidative stress

As previously discussed, the mechanistic pathways responsible for the generation of ROS during IH may be similar to those during IRI because of the comparable pattern of hypoxia and re-oxygenation (Lavie, 2003, Pack and Gislason, 2009). Because recent evidence from experimental animal studies has shown that acute hyperglycaemia augments the generation of ROS and oxidative stress during exposure to ischaemia-reperfusion injury (Hirose et al., 2008, Bemeur et al., 2007, Tsuruta et al., 2010) we wanted to investigate whether this was also true during IH. We felt this was important as over half of T2DM patients suffer from OSA (Einhorn et al., 2007) and thus millions of patients worldwide experience hyperglycaemia and IH simultaneously during sleep.

Therefore, in chapter 6 we investigated whether hyperglycaemia augmented the level of oxidative stress in rats during exposure to repetitive airway occlusions. In contrast to our hypothesis, levels of oxidative stress in the plasma were not greater in animals exposed simultaneously to hyperglycaemia and airway occlusions.

Indeed, neither simultaneous nor independent exposure to these stimuli over six hours was capable of increasing oxidative stress in healthy rats. It is possible that in our study a choice of different biomarkers of oxidative stress than that of nitrotyrosine and MDA could have demonstrated an elevation in oxidative stress. Alternatively, the exposure to IH may have been too brief to evoke a measurable change in oxidative stress.

Although we were unable to demonstrate an elevation in oxidative stress in animals exposed to hyperglycaemia and IH simultaneously, there may be species differences and thus we wished to further investigate this line of research in humans. Furthermore, postprandial hyperlipidaemia is capable of inducing oxidative stress in humans (Ceriello et al., 2002) and following consumption of an evening meal OSA patients especially those with coexisting T2DM are likely to be exposed to combined postprandial hyperglycaemia and hyperlipidaemia simultaneously with IH. Therefore, in chapter 7 healthy humans were exposed to three different conditions; a) IH or b) room air breathing following consumption of a high glucose and lipid meal and c) IH following consumption of a low glucose and lipid meal.

The primary finding in chapter 7 was that simultaneous but not individual exposure to six hours of IH and combined postprandial hyperglycaemia and hyperlipidaemia elicited a significant increase in oxidative stress. These findings may therefore have implications for OSA patients who consume high glucose/lipid meals prior to

sleep, especially those with coexisting T2DM. Although these findings are functionally relevant, conclusions regarding the responsible mechanisms for the elevation in oxidative stress cannot be made because of limitations in the study design. Indeed, because a mixed meal was consumed it was not possible to distinguish between postprandial hyperglycaemia or postprandial hyperlipidaemia being the responsible stimulus for causing the greater oxidative stress during IH exposure or whether a combination of the two was responsible.

In chapter 7, in addition to the interesting results regarding oxidative stress we also demonstrated that IH augments postprandial hyperglycaemia. These results may go some way to explain why several epidemiological studies have demonstrated that OSA is associated with glucose intolerance, insulin resistance and T2DM and that this is independent of other co-morbidities such as obesity (Punjabi et al., 2004, Sulit et al., 2006, Reichmuth et al., 2005). As a result of the absence of plasma insulin measurements, the infrequent blood sampling and mixed meal consumption, the responsible mechanisms for the augmentation in postprandial hyperglycaemia in our study are unknown.

8.4 Respiratory plasticity

8.4.1 Respiratory plasticity in OSA and the importance of CO₂

IH-induced respiratory plasticity is most commonly researched because of the well-established hypothesis that it may promote breathing stability in OSA patients (Mateika and Narwani, 2008). However, there is no evidence to conclusively confirm or refute that respiratory plasticity manifests during sleep in OSA patients. The absence of evidence is primarily due to the lack of suitable durations of unrestricted breathing between apnoeas required to assess levels of normal ventilation and secondly because changes in sleep stage elicit rapid changes in ventilation which may confound results.

In an attempt to overcome these restraints, we used an airway occlusion model of OSA in awake humans and anaesthetised rats (chapter 4 and 6 respectively) to investigate whether ventilatory LTF and/or progressive augmentation of the HVR manifest under these conditions. In chapter 4 we demonstrated that in awake humans respiratory plasticity was expressed following hypoxic airway occlusions but only when P_{ETCO_2} is elevated slightly above normal levels. Additionally, in chapter 6, exposure to airway occlusions without supplemental CO₂ did not elicit respiratory plasticity in anaesthetised rats. Furthermore, we demonstrated the expression of ventilatory LTF in awake humans following intermittent reductions in P_{IO_2} during mild hypercapnia that was abolished within 1-2 minutes of removing

the additional inspired CO_2 . Finally, in chapter 7 we showed that increasing the duration of intermittent reductions in $\text{P}_{\text{I}\text{O}_2}$ to as long as six hours in humans was not capable of evoking respiratory plasticity under pokilocapnic conditions.

Therefore, we have presented strong evidence in this thesis to suggest that IH occurring during repetitive airway obstructions in OSA is likely to elicit activation of mechanisms responsible for the development of respiratory plasticity but its expression may be restrained by low Pa_{CO_2} levels. However, a recent study demonstrated that exposure to intermittent reductions in $\text{P}_{\text{I}\text{O}_2}$ prior to sleep had an effect on upper airway stability in OSA patients during sleep (Yokhana et al., 2012). This suggests that respiratory plasticity may still be expressed during sleep without supplemental CO_2 if it is artificially induced. In that study, OSA patients were exposed to acute IH each evening for 10 days using an almost identical IH protocol to the one we used in chapter 5. Indeed, PET_{CO_2} was elevated above normal levels and similar magnitudes of progressive augmentation of the HVR and ventilatory LTF were expressed to that shown by us. In contrast to the hypothesis that IH-induced respiratory plasticity could help stabilise the upper airway, the AHI increased above baseline following the first daily exposure to acute IH and remained significantly greater than baseline on the final night. Interestingly, they showed that the increase in AHI was correlated to the magnitude of the HVR but did not correlate to ventilatory LTF.

8.4.2 Separating respiratory LTF from progressive augmentation of the HVR

Based on the aforementioned findings by (Yokhana et al., 2012), if respiratory LTF is going to be seriously considered as a therapeutic treatment option for OSA then scientists need to find a way of evoking respiratory LTF independently of progressive augmentation of the HVR. However, a potential stumbling block is that the two phenomena may in fact be the same form of respiratory plasticity demonstrated under different O_2 tensions but mediated by the same mechanisms (Richerson, 2010).

(Fregosi and Mitchell, 1994) previously showed that rats pre-treated systemically with a serotonin antagonist inhibited the development of phrenic and intercostal muscle LTF but not progressive augmentation of phrenic and intercostal muscle activity during intermittent carotid sinus nerve stimulation (i.e. progressive augmentation of the HVR). This strongly suggests that, in cats, different mechanisms might be responsible for the two forms of respiratory plasticity. However, in every human study that has reported the manifestation of progressive augmentation of the HVR and ventilatory LTF, the existence of progressive augmentation of the HVR has been determined by simply comparing minute ventilation of hypoxic episodes at the end of the IH exposure to hypoxic episodes at the start (Harris et al., 2006, Lee et al., 2009, Gerst et al., 2011, Wadhwa et al., 2008, Yokhana et al., 2012). It is possible that the greater minute ventilation during the late hypoxic episodes in these studies and also shown in our study in chapter 5 is simply a result of the greater minute ventilation during euoxia (i.e. it is dependent

of ventilatory LTF). If LTF of phrenic motor nerve activity as mediated by serotonin caused increased ventilation during normoxia it will also cause increased ventilation during hypoxia. In chapter 4 where progressive augmentation of the HVR occurred following hypoxic airway occlusions and in chapter 5 where progressive augmentation of the HVR occurred after IH exposure, ventilatory LTF was also evident. Indeed, without the use of invasive techniques used in animal studies such as intermittent carotid sinus nerve stimulation and application of serotonin antagonists we are not aware of an appropriate study to investigate whether the responsible mechanisms for progressive augmentation of the HVR are different to those of respiratory LTF in humans.

8.4.3 Future use of pharmaceutical aids to mimic IH-induced respiratory LTF

As discussed throughout this thesis there exists strong evidence alluding to IH being the primary stimulus for the development of cardiovascular disease in OSA patients. Therefore, exposing patients to IH with the aim of inducing respiratory plasticity would negate any health benefits gained by alleviating upper airway collapse. However, an enhanced understanding of the cellular mechanisms involved in initiating and maintaining respiratory LTF following IH might enable the development of pharmaceutical aids that evoke respiratory LTF independent of IH exposure. If this is successful and can elicit respiratory LTF without a parallel activation of progressive augmentation of the HVR it could possibly benefit upper airway stability in OSA patients and other conditions associated with respiratory insufficiency. However, even if a pharmaceutical aid can be developed, its success

as a therapeutic treatment may still be dependent on PaCO_2 levels being above the ventilatory threshold. Because evidence already exists to show that IH is beneficial to chronic spinal injury patients without respiratory specific neuronal conditions a pharmaceutical aid mimicking the effects of IH may still be beneficial irrespective of PaCO_2 levels (Dale-Nagle et al., 2010, Lovett-Barr et al., 2012, Trumbower et al., 2012).

Recent research has greatly expanded our understanding of the responsible mechanisms for IH-induced respiratory plasticity in animals. However, until the work performed in this thesis there had not been any studies undertaken to investigate if the same mechanisms are responsible for the development of respiratory LTF in humans. Furthermore, there is convincing evidence in animals that respiratory LTF is pattern sensitive and only IH but not CH can evoke respiratory LTF. However, since the first report of respiratory LTF in awake humans where the requirement for elevating PETCO_2 was demonstrated (Harris et al., 2006) no study had been performed prior to this thesis to investigate whether acute CH also causes respiratory LTF under elevated PETCO_2 conditions. Therefore, in chapter 5 we undertook a study to investigate whether a central or peripheral mechanism was responsible for maintaining ventilatory LTF in humans and whether ventilatory LTF was pattern sensitive in humans as it is in animals.

8.4.4 Hypoxic induced ventilatory LTF is neither pattern sensitive nor maintained by the CB in awake humans

In chapter 5 we demonstrated that in awake humans, exposure to acute IH during sustained mild hypercapnia induced ventilatory LTF, as did an equivalent duration of acute CH. Future, studies are required to examine whether acute CH during sleep also induces ventilatory LTF and importantly whether acute CH induces LTF of upper airway dilator muscle activity.

Evidence from animal studies clearly demonstrates that acute IH but not acute CH induces respiratory LTF and that the mediating mechanisms is serotonin dependent as intermittent but not continuous application of serotonin or 5-HT₂ receptor agonists evokes the same pattern of phrenic LTF (Lovett-Barr et al., 2006, MacFarlane and Mitchell, 2009) and hLTF (Bocchiaro and Feldman, 2004). Our finding that CH elicits ventilatory LTF in humans raises the important question whether different mechanisms may be responsible for respiratory LTF in humans than in animals. Because of this, potential therapeutic solutions that are based on our knowledge from animal research may not be successful in humans. However, in the same study we were able to demonstrate that the ventilatory LTF following acute IH and acute CH was not maintained by a change in CB activity as ventilatory LTF remained during transient exposures to hyperoxia. Therefore, a central mechanism must be responsible although the exact location remains to be determined.

8.5 Future research

8.5.1 Future research into respiratory plasticity

Although it is believed by many that ventilatory LTF might stabilise the upper airway in OSA patients, there is no evidence to support this theory and as shown in chapters 4 and 6, the expression of ventilatory LTF is restrained by low PaCO_2 levels and thus may not manifest in OSA patients. Furthermore, the expression of LTF of upper airway dilator muscle activity that has been shown to occur simultaneously to ventilatory LTF in humans is also restrained by low PaCO_2 levels (Harris et al., 2006). Finally, as discussed previously it may not be possible to induce respiratory LTF without also inducing progressive augmentation of the HVR that was recently shown to augment OSA severity (Yokhana et al., 2012). Therefore, I suggest that future research should focus on a recently reported IH-independent form of upper airway dilator muscle LTF.

In addition to the upper airway muscles receiving neural activation in sync with the central respiratory pattern, they also receive neural activation from a mechanoreceptor reflex that responds to negative upper airway pressure (NUAP) (Akaoshi et al., 2001, Horner et al., 1991). (Ryan and Nolan, 2009b) recently demonstrated that LTF of this mechanoreceptor reflex manifests in rats exposed to modest intermittent UANP. Interestingly, the augmentation in upper airway dilator muscle activity was restricted to episodes of UANP as it was not present during

unrestricted breathing. Furthermore, the addition of IH did not further enhance LTF of the mechanoreceptor reflex or independently induce it. It would therefore appear that intermittent UANP and IH elicit very different forms of LTF. LTF of this mechanoreceptor reflex may have a better chance of evoking a clinical benefit in stabilising the upper airway in OSA patients for two reasons. Firstly, it is unlikely to occur simultaneously with progressive augmentation of the HVR and secondly it is unlikely to be restrained by low PaCO_2 levels because it is not reliant on the chemoreflex. Future research should be performed to investigate the mechanistic pathways of this new form of LTF.

8.5.2 Future research investigating the effects of IH on oxidative stress during hyperglycaemia

In chapter 7 we showed that oxidative stress during IH is augmented following consumption of a high glucose and lipid meal in humans. Future research is required to determine the responsible mechanisms and what are the potential implications to the health of OSA patients with co-existing T2DM.

Studies undertaken to investigate the responsible mechanisms should evoke postprandial hyperglycaemia and hyperlipidaemia separately, so that the stimuli responsible for augmenting oxidative stress during IH can be distinguished. We have previously (chapter 7) proposed a mechanistic pathway involving the enzyme xanthine oxidase that could theoretically mediate a synergistic interaction between

hyperglycaemia and IH. If future research was to show that hyperglycaemia does indeed cause an augmentation in IH-induced oxidative stress the drug allopurinol which inhibits xanthine oxidase activity could be used to investigate our proposed mechanistic pathway. Although human studies of this type would help to determine whether OSA patients with T2DM are at an increased risk of experiencing elevated levels of oxidative stress, animal research is required to determine what effect this may actually have on patients' health.

Although numerous animal studies have been performed to investigate the health effects of chronic exposures to hyperglycaemia or IH, to the best of our knowledge no study exists that has investigated the effects of combined exposures. Future studies using animal models of T2DM should investigate the effects on oxidative stress of simultaneous exposure to nightly IH. Furthermore, these studies could utilise antioxidant treatment to determine if there is a causal relationship between an increase in oxidative stress and the development of cardiovascular disease. In chapters 6 and 7 we only measured oxidative stress in the plasma of rats and humans that gives a whole body measurement of oxidative stress. Although ethical restraints of human research limit these measurements to be made in blood, future animal research should measure oxidative stress in specific tissues to give a better indication of the potential clinical implications of any synergistic interaction between hyperglycaemia and IH.

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